PCT/IL 03/00962

#2

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This is to certify that annexed hereto is a true copy of the documents as originally deposited with the patent application of which particulars are specified on the first page of the annex.

PRIORITY DOCUMENT
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PRIORITY

This 20 -11- 2003 היום

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REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For Receiving Office use only

International Application No. PCT/IL 02/00913

International Filing Date 14 NOV 2002 (14.11.02)

ISRAEL PATENT OFFICE
PCT International Application
Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)* PRO/024 PCT

Box No. I TITLE OF INVENTION

BONE GRAFT COMPOSITE

Box No. II APPLICANT ☐ This person is also inventor

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

PROCHON BIOTECH LTD.
P.O. Box 1482
Rehovot 76114
Israel

Telephone No. 972-8-9310900

Facsimile No. 972-8-9318848

Teleprinter No.

Applicant's registration No. with the Office

State *(that is, country)* of nationality: IL

State *(that is, country)* of residence: IL

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

YAYON, Avner
Moshav Sitria # 104
76834
Israel

This person is:
☐ applicant only
☒ applicant and inventor
☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office

State *(that is, country)* of nationality: IL

State *(that is, country)* of residence: IL

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☐ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

WEBB, Cynthia
Webb & Associates
P.O. Box 2189
Rehovot 76121
Israel

Telephone No. 972-8-9470830

Facsimile No. 972-8-9470860

Teleprinter No.

Agent's registration No. with the Office

☐ **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Box No. V DESIGNATION OF STATES *Mark the applicable check-boxes below; at least one must be marked.*

The following designations are hereby made under Rule 4.9(a):

Regional Patent

☒ **AP** **ARIPO Patent:** GH Ghana, GM Gambia, KE Kenya, LS Lesotho, MW Malawi, MZ Mozambique, SD Sudan, SL Sierra Leone, SZ Swaziland, TZ United Republic of Tanzania, UG Uganda, ZM Zambia, ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

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☒ **EP** **European Patent:** AT Austria, BE Belgium, BG Bulgaria, CH & LI Switzerland and Liechtenstein, CY Cyprus, CZ Czech Republic, DE Germany, DK Denmark, EE Estonia, ES Spain, FI Finland, FR France, GB United Kingdom, GR Greece, IE Ireland, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, PT Portugal, SE Sweden, SK Slovakia, TR Turkey, and any other State which is a Contracting State of the European Patent Convention and of the PCT

☒ **OA** **OAPI Patent:** BF Burkina Faso, BJ Benin, CF Central African Republic, CG Congo, CI Côte d'Ivoire, CM Cameroon, GA Gabon, GN Guinea, GQ Equatorial Guinea, GW Guinea-Bissau, ML Mali, MR Mauritania, NE Niger, SN Senegal, TD Chad, TG Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

National Patent *(if other kind of protection or treatment desired, specify on dotted line)*:

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Check-boxes below reserved for designating States which have become party to the PCT after issuance of this sheet:

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Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation (including fees) must reach the receiving Office within the 15-month time limit.)*

Box No. VI PRIORITY CLAIM

The priority of the following earlier application(s) is hereby claimed:

Filing date of earlier application *(day/month/year)*	Number of earlier application	Where earlier application is:		
		national application: country or Member of WTO	regional application:* regional Office	international application: receiving Office
item (1)				
item (2)				
item (3)				
item (4)				
item (5)				

☐ Further priority claims are indicated in the Supplemental Box.

The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application was filed with the Office which for the purposes of this international application is the receiving Office)* identified above as:

☐ all items ☐ item (1) ☐ item (2) ☐ item (3) ☐ item (4) ☐ item (5) ☐ other, see Supplemental Box

** Where the earlier application is an ARIPO application, indicate at least one country party to the Paris Convention for the Protection of Industrial Property or one Member of the World Trade Organization for which that earlier application was filed (Rule 4.10(b)(ii)):* ....

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used)*:

ISA / US

Request to use results of earlier search; reference to that search *(if an earlier search has been carried out by or requested from the International Searching Authority)*:

Date *(day/month/year)* Number Country *(or regional Office)*

Box No. VIII DECLARATIONS

The following **declarations** are contained in Boxes Nos. VIII (i) to (v) *(mark the applicable check-boxes below and indicate in the right column the number of each type of declaration)*:

Number of declarations

☐ Box No. VIII (i)	Declaration as to the identity of the inventor	:
☐ Box No. VIII (ii)	Declaration as to the applicant's entitlement, as at the international filing date, to apply for and be granted a patent	:
☐ Box No. VIII (iii)	Declaration as to the applicant's entitlement, as at the international filing date, to claim the priority of the earlier application	:
☐ Box No. VIII (iv)	Declaration of inventorship (only for the purposes of the designation of the United States of America)	:
☐ Box No. VIII (v)	Declaration as to non-prejudicial disclosures or exceptions to lack of novelty	:

See Notes to the request form

Box No. IX CHECK LIST; LANGUAGE OF FILING

This international application contains:

(a) the following number of **sheets in paper form:**

request (including declaration sheets)	:	4
description (excluding sequence listing part)	:	39
claims	:	7
abstract	:	1
drawings	:	11
Sub-total number of sheets	:	62
sequence listing part of description *(actual number of sheets if filed in paper form, whether or not also filed in computer readable form; see (b) below)*	:	
Total number of sheets	:	62

(b) sequence listing part of description filed in **computer readable form**

(i) ☐ only (under Section 801(a)(i))

(ii) ☐ in addition to being filed in paper form (under Section 801(a)(ii))

Type and number of carriers (diskette, CD-ROM, CD-R or other) on which the sequence listing part is contained *(additional copies to be indicated under item 9(ii), in right column)*: .....

This international application is accompanied by the following item(s) *(mark the applicable check-boxes below and indicate in right column the number of each item)*:	**Number of items**
1. ☐ fee calculation sheet :	
2. ☐ original separate power of attorney :	
3. ☐ original general power of attorney :	
4. ☐ copy of general power of attorney; reference number, if any: ..... :	
5. ☐ statement explaining lack of signature :	
6. ☐ priority document(s) identified in Box No. VI as item(s): ..... :	
7. ☐ translation of international application into *(language)*: ..... :	
8. ☐ separate indications concerning deposited microorganism or other biological material :	
9. ☐ sequence listing in computer readable form (indicate also type and number of carriers (diskette, CD-ROM, CD-R or other))	
(i) ☐ copy submitted for the purposes of international search under Rule 13*ter* only (and not as part of the international application) :	
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(iii) ☐ together with relevant statement as to the identity of the copy or copies with the sequence listing part mentioned in left column :	
10. ☐ other *(specify)*: ..... :	

Figure of the drawings which should accompany the abstract:

Language of filing of the international application: ENGLISH

Box No. X SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

Cynthia Webb

WEBB, Cynthia
Patent Attorney

For receiving Office use only

1. Date of actual receipt of the purported international application: 14 NOV 2002 (14.11.02)

2. Drawings:
☒ received:
☐ not received:

3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:

4. Date of timely receipt of the required corrections under PCT Article 11(2):

5. International Searching Authority (if two or more are competent): ISA / US

6. ☒ Transmittal of search copy delayed until search fee is paid

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Date of receipt of the record copy by the International Bureau:

Bone Substitute Composite

Field of the Invention

The present invention relates to the field of tissue engineering and more specifically to a composite comprising a synthetic apatite and at least one polymer suitable for use as a bone graft implant, a method of preparing the composite and uses thereof. More particularly the polymer may be natural or synthetic, preferably natural, more preferably a polysaccharide.

Background of the Invention

Tissue engineering may be defined as the art of reconstructing mammalian tissues, both structurally and functionally (Hunziker, Osteoart. Cart., 10:432-465, 2002). In vitro tissue engineering generally includes the delivery of a polymeric or ceramic scaffold that serves as an architectural support onto which cells may attach, proliferate, and synthesize new tissue to replace tissue losses due to disease, trauma or age. Innovations in orthopedic surgery include a vast array of biomaterials that are biocompatible and provide mechanical stability, controlled release of bioactive agents and a scaffold for cell anchorage.

Bone

Bone is a unique type of tissue made up of an inorganic mineral phase and cellular and extracellular matrix phases. By weight, bone contains about 60% mineral material, 30% organic material and the remainder water. Bone has an inherent capacity for repair and regeneration when damaged by disease or trauma but the renewed bone is often fragile and not weight-bearing. Bone restoration or replacement is a viable consideration in indications including osteopenia, osteoporosis, bone tumors, spinal fusion, fractures and non-union fractures.

Many materials have been suggested for bone repair, specifically synthetic materials that avoid the harvesting problems associated with autologous material and the health risks associated with allogenic material. Inorganic material such as calcium phosphate has been utilized as bone and dental fillers (reviewed in LeGeros, Clin Orthop 395:81-98, 2002). Apatite, a particulate calcium phosphate, is particularly appealing by virtue of the fact that it is the naturally occurring mineral component in bone and teeth. Bone apatite exhibits low crystallinity due to the presence of magnesium and carbonate (CO_3) ions. Lack of crystallinity in apatites is associated with increased solubility in vivo. Hydroxyapatite, in contrast, exhibits

high crystallinity and represents a small component of natural bone. Bone substitute materials comprising calcium phosphate or hydroxyapatite have been disclosed for use as bone grafts and implants.

US Patent 4,880,610 teaches a method for producing an injectable calcium phosphate mineral bone-like material using highly concentrated phosphoric acid, a calcium source and a neutralizing source, to which various additives may be incorporated, including sugars or proteins such as collagen, fibrinogen or elastin.

US Patents 5,650,176, 5,676,976 and 5,683,461 teach the synthesis of reactive amorphous calcium phosphates (ACP) and their use for promoting bone growth. US patent 6,214,368 discloses an injectable bone substitute comprising said reactive ACP, an acidic second calcium phosphate material and liquid to form an injectable paste capable of hardening in vivo.

Additional relevant patents describing fluid calcium phosphate compositions that harden include US 5,281,265 and US 6,375,935 which are herein incorporated by reference.

US Patent 5,071,436 discloses a spongy bone substitute matrix consisting of glycosaminoglycans bonded to collagen together with hydroxyapatite.

US Patent 6,118,043 discloses a porous bone replacement material consisting essentially of calcium minerals having an FGF polypeptide contained within.

US Patents 5,650,176, 5,676,976 and 5,683,461 teach the synthesis of reactive amorphous calcium phosphates (ACP) and their use for promoting bone growth.

US Patents 6,027,742 and 6,331,312 disclose a bioceramic composite capable of resorption in the body. The composition comprises resorbable poorly crystalline apatite (PCA) as a cement formed from amorphous calcium phosphate, a promoter and a biocompatible supplementary material selected from bioresorbable polymers or non-resorbable material, which impart a desirable biological, chemical or mechanical property. The composite is prepared by combining the nano size PCA calcium phosphate with the supplementary material.

US patent 6,417,247 provides a composition comprising a polymer or polymer solution that forms a gel under controlled parameters and a ceramic matrix, the composition being fluid under non-physiological conditions and non fluid under physiological conditions. Preferred polymers are polysaccharides, polyamides or polyamino acids, preferred

ceramics are hydroxyapatite (HAP) or tricalcium phosphate ceramics. The compositions are prepared by mixing the ceramic component into a polymer solution.

US Patent 6,231,607 discloses a novel process for the preparation of a bone substitute comprising hydroxy apatite and both α- and β- tricalcium phosphate (TCP), prepared by microwave irradiation and subsequent sintering. The intermediate powder material, resulting from microwave radiation, exhibits strong similarity to natural bone according to X-ray and Fourier transform infrared (FTIR) spectroscopy analyses. The dry powder of that invention enhances bone healing in a rat tibia fracture model. Derivatives or a fluid composition comprising the dry powder were neither taught nor suggested in that disclosure.

There remains a need for material having superior biological and physical properties for use as a bone substitute in orthopedic indications. In particular, there remains a need for a synthetic biocompatible material that mimics the attributes of natural bone that is able to accelerate the rate and enhance the quality of new bone formation. Existing bone graft implants or bone substitute materials are prepared by mixing together various preformed apatites (calcium phosphate materials) with certain polymers or by dispersing pre-formed calcium phosphate materials in the polymers. In general, the calcium phosphate materials or synthetic apatites in the art are prepared using harsh conditions, e.g. low pH (phosphoric acid) or very high temperatures (>450°C), thus precluding the generation of a composite incorporating such polymers during the preparation steps. The art has not heretofore provided a synthetic apatite and polymer composite material wherein the polymer is included ab initio.

Summary of the Invention

It is an object of the present invention to provide a biodegradable and biocompatible bone substitute composite material comprising a synthetic apatite co-crystallized with an amino acid and optionally further comprising at least one polymer. It is another object of the present invention to provide a composite having advantageous biological and physical properties useful in orthopedic, periodontal and craniofacial applications. It is yet another object of the present invention to provide a bone substitute composite that promotes enhanced bone formation in vivo. It is yet another object of the present invention to provide a bone substitute paste comprising the bone substitute composite useful for delivery of bioactive agents to a bone defect or lesion. It is a further object of the present invention to provide a method of using the bone substitute composite. It is yet a further object of the present invention to provide a kit comprising the bone substitute composite.

These and other objects will be apparent from the description, figures and claims that follow.

Although numerous compositions comprising calcium phosphate minerals are known in the art, none has proven entirely satisfactory in meeting the criteria required for successful tissue engineering. The inventors of the present invention have found, quite surprisingly, that a novel composite material is produced by co-crystallizing a liquid mixture of certain polymers and calcium and phosphate ions ab initio. The composite of the present invention provides a superior space filling material to induce local bone formation in orthopedic, periodontal and craniofacial applications where bone formation is required. Furthermore, the composite may be used on its own or as a carrier to deliver bioactive agents to the site of the bone defect or lesion.

According to one currently preferred embodiment of the present invention, a bone substitute composite comprising a synthetic apatite, at least one amino acid in monomeric or polymeric form, a carbonate and at least one additional polymer is provided.

According to one currently more preferred embodiment of the present invention present invention the synthetic apatite is a poorly crystalline apatite (PCA). PCA is regarded as a superior bone replacement material than other synthetic apatite materials such as hydroxyapatite or β-tricalcium phosphate due to its similarity to natural bone and enhanced resorption capacity.

According to another currently preferred embodiment the polymer is introduced during the preparation step of the poorly crystalline apatite ab initio. Without wishing to be bound by

any theory, the presence of the polymer during the formation of the synthetic apatite generates a unique composite material wherein the polymer is intercalated or dispersed or distributed within the crystal structure.

It is now disclosed that the attributes and desirable properties of the bone substitute can be controlled by the addition of at least one polymeric auxiliary agent in the process of forming the poorly crystalline apatite. The composite has attributes that make it particularly advantageous for supporting and promoting bone growth and repair in vivo. Among the advantageous properties of the composite of the invention:

The composite has superior physical properties, controlled by varying polymers used in the preparation. Desirable properties include poor crystallinity that highly resembles that of natural bone, enhanced resorption and convenient use formulation.

The composite has superior biological properties, controlled by varying polymers used in the preparation. Desirable properties include controlled release of bioactive agents, biocompatibility, and the ability to promote cell growth, proliferation, differentiation and migration.

The polymers, which may be natural or synthetic, are selected to impart advantageous attributes to the composite. Without wishing to be bound by theory, the polymers impart cohesive properties to the composite that may be optimized for each of the diverse applications.

The polymers that impart the desired properties are preferably natural rather than synthetic, more preferably natural polysaccharides. Preferred polymers are biocompatible and biodegradable. Examples of synthetic polymers include polyethylene glycol (PEG), polyglycolic acid (PGA), polyanhydrides, polylactic acid (PLA), poly-L-lactic acid (PLLA) and their co-polymers, polyamides, block copolymers or combinations thereof. Examples of natural polymers include polysaccharides, oligosaccharides and polyamino acids. Examples of polysaccharides include dextran, polylactate and polyglycolic acid.

Currently preferred polysaccharides are sulfated polysaccharides, currently more preferred are glycosaminoglycans including chondroitin 4-sulfate, chondroitin 6-sulfate, hyaluronic acid, dermatan sulfate, keratan sulfate, heparin, heparan sulfate, sucrose octasulfate, perlecan, syndecan, glypican and combinations thereof. Heparin is meant to include the multiple molecular weight derivatives of heparin including very low molecular weight heparin, low

molecular weight heparin, heparan, and heparin mimetics. Additional natural polymers include starch, collagen, gelatin, glycogen, chitin, cellulose, keratins or combinations thereof.

According to one currently more preferred embodiment the present invention provides a composite comprising a synthetic apatite, an amino acid in monomeric or polymeric form, a carbonate, and at least one polymer, further comprising at least one bioactive agent selected from the group consisting of antibiotics, antiviral agents, chemotherapeutic agents, anti-rejection agents, analgesics and analgesic combinations, anti-inflammatory agents, hormones, growth factors and cytokines. Preferably the at least one bioactive agent is a growth factor. According to one currently most preferred embodiment of the present invention the at least one bioactive agent is selected from the group consisting of fibroblast growth factors (FGF) and their variants. Preferably a therapeutically effective amount of FGF is provided, said FGF having the capacity to induce bone growth and or angiogenesis.

These and other features result in a composite exhibiting advantageous properties including biocompatibility, biodegradability, osteoconductivity and osteoinductivity, and ease of administration.

The bone substitute composite is further characterized in that it comprises a synthetic apatite more preferably poorly crystalline apatite (PCA) having crystallinity similar to that of natural bone. Preferably the composite has an X-ray diffraction pattern similar to that of poorly crystalline apatite and natural bone. More preferably the composite exhibits an undifferentiated peak of 2 theta (2θ) in the 31°-33° range.

The bone substitute composite further exhibits a calcium to phosphate ratio (Ca/P) similar to that of natural bone. Accordingly, the synthetic apatite may contain cation or anion substitutions. According to one currently preferred embodiment, magnesium ions (Mg^{++}) and/or zinc (Zn^{++}) are added to partly replace the calcium ions, preferably Mg^{++}.

The bone substitute composite may be administered as a powder for certain bone disease and injury applications. In certain indications a fluid or semi-fluid composition is preferred.

According to one preferred embodiment of the present invention a pharmaceutical composition comprising the bone substitute composite is provided. The composition may be fluid or semi-solid. According to one more preferred embodiment of the present invention the composition is paste-like. In preferred embodiments the composition is an injectable paste. Viscosity of the injectable paste is preferably in the range of 10-500 poises, more preferably in the range of 30-200 poises, depending on the application.

According to another preferred embodiment of the present invention the pharmaceutical composition is fluid at temperatures below physiological conditions and non-fluid at physiological temperatures. Preferably the composition gels or hardens at between 35°-42°C. This particular property of the composition may be achieved by the addition of certain polymers or other additives to the composite of the invention. Preferably the composition comprises an additive that promotes in situ hardening of said composition over about 10-60 minutes following injection. A non-limiting example of such materials includes collagens such as a gel forming soluble collagen disclosed in WO 00/47130, and non-polymeric compounds such as a non-polymeric esters or mixed esters of one or more carboxylic acids disclosed in US Patent 6,413,536. Another non-limiting example includes the addition of calcium sulfate or calcium phosphate compounds. The composition may comprise about 5-50% calcium sulfate.

Alternatively, in the reconstruction of structural tissues like cartilage and bone, certain applications may require the in vitro molding of the composition into three dimensional configuration articles of varying thickness and shape. Accordingly, provided is an implant comprising the composite of the invention further comprising a hardener, said implant having a specific shape including a sphere, screw, cube, rod, tube or plate.

In preferred embodiments of the present invention a pharmaceutical composition is provided comprising a composite material comprising synthetic apatite, at least one amino acid in monomeric or polymeric form, at least one additional polymer, optionally further comprising at least one bioactive agent, further comprising a pharmaceutically acceptable carrier or diluent, optionally further comprising a hardener.

According to one currently preferred embodiment of the present invention a pharmaceutical composition is provided comprising a synthetic apatite and heparin composite, at least one carrier having sufficient fluidity to enable injection of the composition to the site of treatment. According to one currently more preferred embodiment of the present invention a pharmaceutical composition is provided comprising a synthetic apatite and heparin composite, at least one carrier having sufficient fluidity to enable injection of the composition to the site of treatment and a therapeutically affective amount of at least one bioactive agent selected from the group consisting of growth factors and their variants. According to one currently most preferred embodiment of the present invention the at least one bioactive agent is selected from the group consisting of fibroblast

growth factors (FGF) and their variants. Preferably the FGF is an FGF having the capacity to induce bone growth and or angiogenesis.

The pharmaceutical composition of the present invention is useful for treating orthopedic, periodontal and craniofacial indications wherein there is need to fill a void in a bone or a need to delivery bioactive agents to the bone or tissue in contact with the bone. Tissue closely associated with bone includes, ligaments, tendons cartilage and muscle. In accordance with the invention provided is the use of the composite of the invention for the manufacture of a medicament for treating diseased or injured bone in orthopedic, periodontal and craniofacial indications wherein the composite is provided alone or comprising bioactive agents that accelerate the healing rate and enhance the quality of bone formation or treat a disease or traumatized bone associated tissue.

According to one aspect of the present invention the synthetic apatite of the composite is a poorly crystalline apatite. According to one exemplary embodiment the composite is prepared by microwaving the calcium and phosphate ions with the polymer using a procedure disclosed in US Patent 6,231,607. The powder resulting from that process consists of poorly crystalline apatite (PCA) calcium phosphate aggregates having a size of approximately 0.45 μm to 6μm in diameter, more preferably 1 μm-4 μm in diameter. The aggregates are comprised of crystals of approximately 0.20 μm-0.30 μm in size.

Another embodiment of the present invention provides a process for the preparation of a fluid bone composition comprising the poorly crystalline apatite according to US Patent 6,231,067 further comprising adding at least one additional polymer, and optionally adding at least one bioactive agent. The process for preparing the composition comprises sterilizing the powder, adding a sufficient amount of liquid to hydrate and disperse the powder, adsorbing a bioactive agent and preparing the wetted powder for administration. Following the wetting procedure the composition may be optionally filtered to remove excess liquid, thus leaving a paste like material on the filter.

The process comprises the following steps:

a) preparing a liquid mixture comprising ionic calcium, phosphate, at least one amino acid in either monomeric or polymeric form, carbonate, comprising at least one additional polymer, optionally further comprising a bioactive agent;
b) subjecting said mixture to microwave irradiation;
c) quenching said irradiated mixture;

d) filtering said irradiated mixture so as to separate between the filtrate and a cake;

e) drying said cake;

f) grinding said dried cake into a powder;

g) sterilizing said powder;

h) wetting said sterilized powder with a solution optionally comprising at least one bioactive agent;

i) preparing said wetted powder for administration.

According to one currently preferred embodiment of the present invention the powder resulting from step (f) consists of poorly crystalline apatite (PCA) calcium phosphate aggregates having a size of approximately 0.45 μm to 6μm in diameter, more preferably 1 μm-4 μm in diameter. The aggregates are comprised of crystals of approximately 0.20 μm-0.30 μm in size.

According to one currently preferred embodiment of the present invention the PCA powder composite resulting from step (f) is sterilized in a manner that substantially retains the X-ray diffraction pattern of the powder, preferably by ionization techniques, more preferably by γ-irradiation. The present inventors have found that following sterilization by γ-irradiation the bone substitute composite retains its molecular crystal structure, as determined by X-ray diffraction analysis. Preferably the composite retains an X-ray diffraction pattern having an undifferentiated peak of 2 theta=31°-33°

Thermal treatment of certain calcium phosphate compositions at very high temperatures e.g. <450°C results in increased crystallinity, i.e. production of a ceramic that is essentially non-resorbable in vivo. The inventors of the present invention demonstrate that thermal sterilization treatment of the PCA powder resulting from step (f) at lower temperatures results in a minor change in the X-ray diffraction spectrum. The change is seen in the X-ray diffractogram wherein the undifferentiated peak of 2θ=31°-33° has two distinguishing reflections which may be seen at approximately 2θ=32.1° and 32.7°. These reflections indicate the formation of a more organized structure. The thermal treatment comprises placing the PCA powder in a dry heat oven, of about 140°-160°C for at least 30 minutes, more preferably 2 hours. According to one currently preferred embodiment of the present invention, a composition comprising a thermal sterilization step, wherein said sterilization is carried out at about 140°-160°C for at least 30 minutes is useful as bone substitute.

A currently preferred embodiment of the present invention provides a pharmaceutical composition comprising a sterilized synthetic apatite powder together with a pharmaceutically acceptable liquid such as water or a physiological fluid. The liquid is added in a sufficient amount to permit wetting and dispersion of the powder to form a wetted mixture having the consistency of a paste or putty. The liquid may advantageously comprise at least one bioactive agent selected from antibiotics, antiviral agents, anti-rejection agents, analgesics and analgesic combinations, anti-inflammatory agents, hormones, growth factors, cytokines and chemotherapeutic agents including anti-cancer compounds and compounds that prevent bone resorption.

The bioactive agents, for example, growth factors, angiogenic factors, and the like, are advantageous to encourage a more rapid growth of the cells within the implant, or a more rapid vascularization of the implant. Such factors may be too small to be effectively retained within the matrix and hence are introduced in the form of slow-release or controlled-release formulations into the matrix to provide for their effectiveness. Heparin is known to be a stabilizer of certain growth factors and a system for stabilizing fibroblast growth factors by binding them to certain substrates via heparin or heparin derived compounds is disclosed in US Patent No. 5,100,668.

According to a preferred embodiment, a sufficient amount of liquid is added to permit wetting and dispersion of the powder to form a hydrated precursor mixture having a consistency compatible with application to a filtration device. The wetted powder is filtered through a sterile filtration device having pore size enabling retention of the crystalline aggregates on the filter. Preferably, the pore size of the filtration device permits full retention of the bone substitute material.

The filtered material retains sufficient fluidity to enable handling without fragmentation or separation of the liquid from the solid phase. Preferably the wetted powder has a consistency or putty or paste. Viscosity of the injectable paste is preferably in the range of 10-500 poises, more preferably in the range of 30-200 poises, depending on the application.

In another embodiment of the present invention, the wetted powder is blended under sterile conditions to a consistency compatible with administration to a site of a defect or lesion. The paste may be administered manually or with a spreading instrument such as a spatula.

More preferably, the wetted powder is inserted into a syringe and is prepared for local administration or injection into the site of the defect or lesion.

In one currently preferred embodiment of the present invention the powder is mixed with liquid comprising a growth factor, in particular an FGF or FGF variant the powder and the liquid being mixed at a w/w or w/v ratio of about 1:1 to yield a paste-like pharmaceutical composition. In one particular exemplary embodiment 3 gm powder is mixed with 3 ml sterile aqueous solution to yield approximately 5ml paste-like composition.

In another particular exemplary embodiment 3 gm PCA powder are mixed with 20 ml sterile aqueous solution and filtered through a 0.45 μm filter to remove excess liquid to yield approximately 5 ml paste-like material.

In yet another embodiment the paste-like material is prepared for administration. According to one currently preferred embodiment the paste-like material is administered directly to a bone defect. According to one currently more preferred embodiment the paste-like material is inserted into a syringe for local administration. According to one currently most preferred embodiment the paste-like material hardens in situ in about 10-60 minutes following injection. Alternatively the paste-like material hardens in vitro to form a molded implant. Furthermore, the composite may be used as a coating on synthetic or other implants such as pins and plates, for example, in hip replacement procedures. Thus, the present invention further provides implants or medical devices coated with the matrix of the invention.

According to yet another currently preferred embodiment of the present invention provides a kit comprising the disclosed bone graft composition, where the dry and liquid components may be present in separate containers in the kit, or some of the components may be combined into one container.

Brief Description of the Figures

Figure 1 shows the X-Ray diffraction patterns of the bone substitution composites of the invention.

Figure 2 shows the particle forms of the bone substitution composites as seen in SEM (scanning electron microscope). Figure 2A shows bone substitute material without additives. Figure 2B shows bone substitute-heparin (0.5ug/ml) composite, Figure 2C shows bone substitute-heparin (100ug/ml) composite. All figures are 39570X magnification.

Figure 3 shows the results of the adsorption (figure 3A) and proliferation (figure 3B) assays for FGF2v. Adsorption was tested to PCA and PCA composites. Proliferation was tested on FGFR1 expressing FDCP cells with FGF2v released from PCA and PCA composites.

Figure 4 shows a histological section of bone repair in a rat tibia model.

Figure 5 shows a histological section of bone repair wherein PCA adsorbed with a growth factor was assayed in a rat tibia model

Detailed Description of the Invention

The present invention is directed to a biocompatible composite useful as a bone substitute implant.

In principle, an ideal bone substitute will exhibit the following properties:

Biocompatible: minimal toxicity and maximal resemblance to natural bone.

Osteoconductive: provide a microenvironment beneficial to attachment, migration and proliferation of cellular elements involved in bone growth.

Resorbable: capacity to biodegrade in the host over time.

Osteoinductive: capacity to enhance regeneration of functional bone.

Practical: convenient for use by the medical practitioner.

The present invention provides a composition exhibiting the aforementioned advantageous properties.

Definitions

For convenience and clarity certain terms employed in the specification, examples and claims are described herein.

The term "biocompatible" as used herein refers to materials having low toxicity, affinity with living tissues and no unacceptable foreign body reactions in the living body.

The term "osteoconductive" as used herein refers to materials that provide a microenvironment that is advantageous to the healing of diseased or damaged bone. Preferably the composite of the invention provides a milieu that is advantageous to the infiltration and proliferation of cells involved in the process of bone repair.

A "composite" as used herein refers to a material that is made up of two or more distinct elements. The composite of the invention is unique in that it comprises a mineral phase and an organic phase that are intercalated or interdispersed ab initio.

A composite or composition considered to be "resorbable" is soluble and degrades in vivo. Preferably the material remains intact throughout the initial healing of the bone and degrades slowly as the host's cells invade and proliferate within the area of the implant. "Biodegradable" refers to the resorption of the composite or composition within the host in a manner that is non-toxic and non-immunogenic to the host.

The term "fluid" as used herein is intended to describe a composition having sufficient viscosity so as not to disperse from the space it is intended to fill, yet having a viscosity low enough to be able to be administered via syringe.

The term "viscosity" refers to the property of resistance to flow in a fluid or semi-fluid. Viscosity is measured in a unit known as a poise. Suitable viscosities of the final solution mixture of the pharmaceutical composition for each particular application may readily be established by the skilled person, but will generally be in the range of about 10 to about 500 poises, preferably about 30 to about 200 poises.

This term "implantation" refers to the insertion of the composition of the invention into a patient, whereby the implant serves to replace, fully or partially, tissue that has been damaged or removed. Another aspect of implantation is also taken to mean the use of the composite as a vehicle to transport therapeutic agents to a certain site in a patient. In this aspect there is also included the adsorption onto the composite or of a bioactive agent selected from growth factors, cytokines, chemotherapeutic drugs, enzymes, anti-microbials, anti-inflammatory agents. A fluid, semi-fluid or solid material may be implanted.

The term "injection" refers to the insertion of a composition of the invention into a mammal using a syringe or other device which allows administration of the composition directly to the site of treatment. The composition serves to replace, fully or partially, tissue that has been damaged or removed. In particular, the composition of the invention is intended to fill a void in a bone, due to disease such as osteopenia or osteoporosis or to damage such as a fracture or non-union. Another aspect of injection is also taken to mean the use of the composite as a vehicle to transport therapeutic drugs and bioactive agents to a certain site in a patient. In this aspect there is also included the introduction into the composite of a bioactive agent selected from growth factors, cytokines, enzymes, anti-microbials, anti-inflammatory agents, chemotherapeutic agents such as anti-cancer drugs or bone resorption preventing drugs.

The bioactive agents, for example, growth factors, angiogenic factors, and the like, are advantageous to encourage a more rapid growth of the cells within the composite, or a more rapid vascularization of the material thus reducing the healing time. Such factors may be too small to be effectively retained within the composition and hence may be

introduced in the form of slow-release or controlled-release formulations into the composite to provide for their effectiveness.

Chemotherapeutic agents include a variety of chemical compounds that prevent or treat bone resorption disorders or diseases including but not limited to bisphosphonates.

Polymers

According to one currently preferred embodiment of the present invention the composite comprises polymeric agents that modulate the physical and biological properties including crystallinity, surface adhesion, cohesion, ability to maintain cell growth and proliferation, and binding and retention of bioactive agents, said bioactive agents including but not limited to proteins, polypeptides and drugs.

These polymers include materials belonging to the family of polysaccharides, anionic polysaccharides, glycosaminoglycans, or synthetic polymers, including hyaluronic acid, pectin, alginate, galactans, galactomannans, glucomannans, polyuronic acids, heparin, dextran sulfate, dermatan sulfate, heparin, heparan sulfate, keratan sulfate, hexuronyl hexosaminoglycan sulfate, chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate, keratan sulfate, perlecan, syndecan, glypican and PEG and combinations thereof.

Preferably the composite is prepared with polymers such as heparin or heparin mimetics. The inventors of the present invention have discovered a novel composite comprising a synthetic apatite and polymer wherein the polymer is included ab initio

"Heparin mimetics" as used herein includes polysulfated sugars, such as polysulfated monosaccharides, polysulfated disaccharides and polysulfated oligosaccharides, including sucrose octasulfate, inositol hexasulfate, and many other polysulfated sugars that may act as analogs of low molecular weight heparins may be used to substitute for sulfated polysaccharides. For instance, US 6,143,730 discloses sulfated oligosaccharides comprising from 3 to eight monosaccharide units.

An "anionic polysaccharide" as used herein, is a polysaccharide, including non-modified as well as chemical derivatives thereof, that contains at least one negatively charged group (e.g., sulfate groups which are negative at neutral pH, and carboxyl groups at pH values above about 4.0) and includes salts thereof, such as sodium or potassium salts, alkaline earth metal salts such as calcium or magnesium salts. Non-limiting examples of anionic polysaccharides include pectin, alginate, galactans, galactomannans, glucomannans and polyuronic acids.

Non-limiting examples of sulfated polysaccharides include heparin, chondroitin sulfate, dextran sulfate, dermatan sulfate, heparan sulfate, keratan sulfate, hexuronyl hexosaminoglycan sulfate, inositol hexasulfate, and sucrose octasulfate. Derivatives and mimetics of the above are intended to be included in the invention.

Another embodiment of the invention provides a composite comprising the synthetic apatite and a polymer further comprising one or more bioactive agents including drugs such as antibiotics and antiviral agents; chemotherapeutic agents; anti-rejection agents; analgesics and analgesic combinations; anti-inflammatory agents; hormones such as steroids and growth factors such as fibroblast growth factor. Further provided by the present invention is a composition comprising the composite impregnated with a drug or agent able to deliver high tissue levels of said drug to the site of injured or diseased bone or to a tissue associated with bone. A composite of this type is particularly useful for, but not limited to, delivering antibiotic therapy to osteomyelitis patients and bisphosphonate therapy to patients with multiple myeloma and osteoporosis.

According to another aspect of the invention, the bioactive agents include cytokines, growth factors and their activators etc, for example, in order to enhance a therapeutic effect or to provide a slow-release or sustained-release mechanism. For example, growth factors, structural proteins or cytokines which enhance the temporal sequence of bone repair, alter the rate of proliferation or increase the metabolic synthesis of extracellular matrix proteins are useful additives to the composite of the present invention. Representative proteins include bone growth factors (BMPs, IGF) including BMP2 and BMP7 and fibroblast growth factors, including FGF2, FGF4, FGF9 and FGF18 and variants thereof for bone and cartilage healing. Other factors shown to act on cells forming bone include retinoids, growth hormone (GH), leptin and transferrin.

The proteins of the invention are polypeptides or derivatives, muteins or variants thereof, obtained from natural, synthetic or recombinant sources, which exhibit the ability to stimulate DNA synthesis and cell division in vitro of a variety of cells, particularly cell types involved in bone regeneration and remodeling. A non-limiting example of FGF variants is disclosed in WO 02/36732.

Additionally, cells genetically engineered to express the aforementioned proteins are including in the present invention. Preferred examples for bone repair uses periosteal or other mesenchymal stem cells or osteocytes/osteoblasts per se or transfected with bone

growth factor genes selected from a group including bone morphogenetic protein (BMP) family genes or fibroblast growth factor (FGF) family genes. According to one currently preferred embodiment of the present invention the composite comprises at least one growth factor of the FGF family having osteoinductive activity. According to one currently more preferred embodiment of the present invention the composite further comprises a growth factor of the BMP family.

Other bioactive agents intended to be incorporated in the present invention include blood factors that regulate clot formation such as fibrin and plasminogen.

The mineral component of bone is predominantly made of calcium phosphates. "Hydroxy apatite" refers to a highly crystalline calcium phosphate having the chemical formula: $Ca_5(PO_4)_3OH$. Hydroxy apatite and other highly crystalline synthetic apatite materials are considered to be less than optimal bone substitute implants since, although they are biocompatible, they are for the most poorly biodegradable. The mineral fraction of natural bone is primarily composed of "poorly crystalline apatite", a calcium phosphate derivative. It is to be understood that that a part of the calcium (Ca^{++}) ions may be replaced with other divalent ions selected from the group consisting of Magnesium (Mg^{++}) and Zinc (Zn^{++}). The incorporation of additional or different divalent ions imparts on the composition certain properties which may be advantageous to bone repair and growth.

In preferred embodiments of the present invention a pharmaceutical composition comprising a synthetic apatite and a polymer composite further comprising a pharmaceutically acceptable carrier or excipient is provided. According to one currently preferred embodiment of the present invention the pharmaceutical composition further comprises at least one bioactive agent. According to one currently more preferred embodiment of the present invention a pharmaceutical composition comprising a synthetic apatite and heparin composite, at least one carrier having sufficient fluidity to enable injection of the composition to the site of treatment and at least one bioactive agent selected from the group consisting of growth factors and their variants is provided. According to one currently most preferred embodiment of the present invention the at least one bioactive agent is selected from the group consisting of fibroblast growth factors and their variants.

The pharmaceutical composition of the present invention is useful for treating orthopedic, periodontal and craniofacial indications wherein there is need to fill a void in a bone or a

need to delivery bioactive agents to a bone lesion or defect. In accordance with the invention, provided is the use of the composite of the invention for the manufacture of a medicament for treating diseased or injured bone in orthopedic, periodontal and craniofacial indications wherein the composite is provided alone or comprising bioactive agents that accelerate the healing rate and enhance the quality of bone formation.

In certain applications, a solid implant is desired. Further provided is a bone substitute composition comprising a synthetic apatite, at least one amino acid in monomeric or polymeric form, further comprising at least one polymer, optionally further comprising a bioactive agent, further comprising an additive that promotes hardening of said composition in situ over about 10-60 minutes following injection. Alternatively, in the reconstruction of structural tissues like cartilage and bone, certain applications may require implantation of a solid implant. This may be achieved by molding of the composition into three dimensional configuration articles of varying thickness and shape in vitro. Accordingly, provided is an implant comprising a synthetic apatite, at least one amino acid in monomeric or polymeric form, further comprising at least one polymer, optionally further comprising a bioactive agent, further comprising an additive that promotes hardening of said composition which may be formed to assume a specific shape. The shapes include a sphere, cube, rod, tube or a sheet which may constitute a prosthesis. A non-limiting example of a hardener includes calcium sulfate or calcium phosphate compounds. The shape is determined by the shape of a mold or support which may be made of any inert material and may be in contact with the composite on all sides, as for a sphere or cube, or on a limited number of sides as for a sheet.

Poorly Crystalline Apatite Composition

According to an alternative embodiment, the synthetic apatite prepared according to US Patent 6,231,067 is now shown to be useful as a carrier for delivering bioactive agents to the site of bone disease or injury. Bioactive agents, one or more bioactive agents including drugs such as antibiotics and antiviral agents; chemotherapeutic agents; anti-rejection agents; analgesics and analgesic combinations; anti-inflammatory agents and hormones such as steroids. Further provided by the present invention is a pharmaceutical composition comprising the synthetic apatite impregnated with a drug or chemical able to deliver high tissue levels of said drug to the site of injured or diseased bone. A pharmaceutical composition of this type is particularly useful for, but not limited to, providing an osteoinductive growth factor to a fracture, delivering local antibiotic therapy to

osteomyelitis patients and delivering local bisphosphonate therapy to patients with osteoporosis or lesions resulting from multiple myeloma.

According to other another aspect of the invention, the bioactive agents include cytokines, growth factors and their activators etc, as described above. The proteins of the invention are polypeptides or derivatives, muteins or variants thereof, obtained from natural, synthetic or recombinant sources, which exhibit the ability to stimulate DNA synthesis and cell division in vitro of a variety of cells, particularly cell types involved in bone regeneration and remodeling.

Additionally, cells genetically engineered to express the aforementioned proteins are including in the present invention. Preferred examples for bone repair uses periosteal or other mesenchymal stem cells or osteocytes/osteoblasts per se or transfected with bone growth factor genes selected from a group including bone morphogenetic protein (BMP) family genes or fibroblast growth factor (FGF) family genes. According to one currently preferred embodiment of the present invention the composite comprises at least one growth factor of the FGF family having osteoinductive activity. According to one currently more preferred embodiment of the present invention the composite further comprises a growth factor of the BMP family.

Other bioactive agents intended to be incorporated in the present invention include blood factors that regulate clot formation such as fibrin and plasminogen.

According to one currently preferred embodiment of the present invention a composition comprising said synthetic apatite is prepared for administration comprising sterilizing the powder, adding a sufficient amount of liquid to hydrate and disperse the powder, and preparing the wetted powder for administration. Following the wetting procedure the composition may be optionally filtered to remove excess liquid, thus leaving a paste-like material on the filter.

The process comprises the following steps:

a) preparing a liquid mixture comprising ionic calcium, phosphate, at least one amino acid in either monomeric or polymeric form, carbonate, further comprising at least one additional polymer, optionally comprising a bioactive agent;
b) subjecting said mixture to microwave irradiation;
c) quenching said irradiated mixture;
d) filtering said irradiated mixture so as to separate between the filtrate and a cake;

e) drying said cake;

f) grinding said dried cake into a powder;

g) sterilizing said powder;

h) wetting said sterilized powder with a solution optionally comprising at least one bioactive agent;

i) preparing said wetted powder for administration.

The mixture of step a) comprises a calcium ion that may be, for example, calcium chloride added to a concentration of about $5x10^{-3}$ to about $5x10^{-2}$. The preferred concentration is about $1x10^{-2}$. The phosphate may be a phosphate such as NaH_2PO, added to a concentration of about $3x10^{-3}$ to about $2x10^{-2}$. The preferred concentration is about $6x10^{-3}$. The amino acid may be any monomeric or polymeric amino acid but is preferably L-aspartic acid, added to a concentration of about 10-50 ppm. The preferred concentration is about 25 ppm. The carbonate may be, for example $NaHCO_3$, added to a concentration of about 1-600 ppm. The preferred concentration of carbonate is about 150 ppm.

According to one currently preferred embodiment of the present invention the powder resulting from step (f) consists of poorly crystalline apatite (PCA) calcium phosphate aggregates having approximately 0.45 μm to 6 μm in diameter, more preferably 1 μm-4 μm in diameter.

The microwave step is typically carried out in a standard kitchen 700W-1000W microwave for approximately 10-30 minutes.

The powder in its dry form, following autoclave and drying, was shown to be a graft material for filling holes in bones (Ben-Bassat et al, in Biomaterials Engineering and Devices: Human Applications v2, p155-169, 2000). The present invention provides a unique composite with superior properties for use as a bone substitute in indications where a space filling material is needed.

The composition should be sterilized for use in vivo, in particular for use in clinical and therapeutic applications in mammals. The present inventors have shown that the dry PCA powder is sterilizable by ionization, preferably γ-irradiation, and retains its original X-ray diffraction pattern. The powder resulting from step (f) was irradiated at a minimum of 2.5 Mrad according to known GMP production procedures, followed by X-ray diffraction analysis.

Following thermal sterilization, e.g. 140°C for 30 minutes to 2 hours, the X-ray diffraction pattern is altered slightly and shows two distinguishing reflections which may be seen at approximately 2θ=32.1° and 32.7°. These reflections indicate the formation of a more organized structure. According to one currently preferred embodiment of the present invention, this composition is useful as bone substitute.

A currently preferred embodiment of the present invention provides wetting the sterilized PCA powder with a pharmaceutically acceptable liquid such as water or a physiological fluid preferably comprising a growth factor other bioactive agent. The liquid is added in a sufficient amount to allow wetting and dispersion of the powder to form a wetted mixture having the consistency of a paste or putty. In one currently preferred embodiment of the present invention the powder is mixed with liquid in a ratio of about 1:1 w/w or w/v to yield a paste-like substance. In one particular exemplary embodiment 0.3 gm powder is mixed with 0.3 ml sterile water comprising growth factor, in particular an FGF or FGF variant to yield approximately 0.5ml paste-like composition.

Alternatively, a sufficient amount of liquid is added to permit wetting and dispersion of the powder to form a hydrated precursor mixture having a consistency compatible with application to a filtration device. The wetted powder is filtered through a sterile filtration device having pore size enabling retention of the crystalline aggregates on the filter. Preferably, the pore size of the filtration device permits full retention of the bone substitute material. In one particular exemplary embodiment 0.3 gm PCA powder are mixed with 2 ml sterile PBS comprising growth factor, in particular an FGF or FGF variant. The mixture was left for 1 hour to allow the FGF to adsorb to the PCA and the slurry filtered through a 0.45 μm filter to remove excess liquid to yield approximately 0.5 ml paste-like material.

The filtered material is left sufficiently wetted in order to enable handling without fragmentation or crumbling or separation of the liquid from the solid phase. Preferably the wetted powder has a consistency or putty or paste. Preferably the paste has a viscosity in the range of 10-500 poises, more preferably 30-200 poises.

In another embodiment of the present invention, the wetted powder is blended under sterile conditions to a consistency compatible with administration to a lesion. The paste may be administered manually or with a spreading instrument such as a spatula. More preferably, the wetted powder is inserted into a syringe and is prepared for local administration or injection into the site of the defect or lesion.

Bone Repair

Fractures and other defects in long bones heal via a process known as endochondral ossification while defects and lesions in intramembranous bones heal via an osteogenic route (Rabie, et al., Int J Oral Maxillofac Surg 25(5):383, 1996). Four stages of fracture repair have been characterized (reviewed in Bolander, Proc. Soc. Exp. Biol. Med. 200(2): 165, 1992). Stage 1 is the immediate injury response; stage 2 marks the synthesis of new bone matrix and callus formation in a process termed intramembranous ossification; stage 3, designated chondrogenesis, occurs as the mesenchymal cells develop into chondrocytes and are eventually replaced by cartilage; stage 4 is the formation of bone from cartilage in a process known as endochondral ossification.

According to the principles of the present invention the composite of the invention may be used in additional orthopedic indications including periodontal surgery, and plastic and craniofacial surgery. In a non-limiting example, the composite of the present invention may be used for augmentation of the alveolar ridge to facilitate retention of denture and to fill various periodontal lesions. It can also be used in to fill the gap in cases of bony defects, cysts and traumatic bone loss. The composite of the present invention may be used for maxillofacial dysplasia, filling of bone defects in skull, zygomatic and mandibular area and augmentation of various bony areas. In addition, the composite of the present invention may be used to reconstruct the calvaria including repair of cranial base and temporal bone defects following surgery. Orthopedic applications in which the compositions of the invention are useful include, but are not limited to, fractures and non-union fractures resulting from a trauma or generated by surgical means, spinal fusion, hip resurfacing or bone augmentation in indications such as osteopenia or osteoporosis.

According to the principles of the present invention the composite comprises bioactive agents that have the capacity to act at some or all of the stages in order to enhance bone repair and ensure the formation of functional bone.

Pharmacology

The term "therapeutic" refers to any pharmaceutical, drug or prophylactic agent which may be used in the treatment (including the prevention, diagnosis, alleviation, or cure) of a malady, affliction, disease or injury in a patient.

The term "excipient" as used herein refers to an inert substance added to a pharmaceutical composition to further facilitate administration of a compound. Examples, without

limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, or gelatin. Pharmaceutical compositions may also include one or more additional active ingredients.

Pharmaceutical compositions of the present invention may be manufactured by processes well known in the art, e.g., by means of conventional mixing, dissolving, granulating, grinding, pulverizing, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

The term "physiologically acceptable liquid carrier" or "diluent" refers to an aqueous or non-aqueous fluid that is well suited for injection preparations.

The composite of the experiment may be used in particle or powder form, or may be combined with a physiological liquid for use as a paste-like material. The composition may further comprise hardening agents for in situ or in vivo hardening that results in a molded body.

The pharmaceutical composition of this invention may be administered as a paste, preferably as an injectable paste, more preferably as an injectable paste that hardens in situ, in about 10-60 minutes following implantation. Alternatively an implant comprising the composite of the invention is provided. Furthermore, the composite may be used as a coating on synthetic or other implants such as pins and plates, for example, in hip replacement procedures. Thus, the present invention further provides implants or medical devices coated with the matrix of the invention.

According to an alternative embodiment the pharmaceutical composition further comprises at least one agent that renders the composition non-fluid under physiological conditions. A non-limiting example of a collagen that gels at physiological temperatures is disclosed in WO 00/47130.

Kits

The present invention further provides a kit comprising the disclosed bone graft composite, where the dry and liquid components may be present in separate containers in the kit, or some of the components may be combined into one container.

Further provided is a kit comprising the poorly crystalline apatite, where the dry and liquid components may be present in separate containers in the kit, or some of the components may be combined into one container.

Examples

Example 1: Preparation of Bone graft powder

A method for preparation of the bone substitute is disclosed in US patent 6,231,607. The bone substitute prepared by this method has an X-ray diffraction pattern similar to natural bone. The inventors now disclose that polymers may be added during the formation of the poorly crystalline apatite crystals. The powder can be formulated into a fluid composition having advantageous properties for use as a bone graft material.

Materials and Methods

Graduated bottles, 2-liter capacity and 500 ml capacity

Glass ice bath

Microwave Oven (700W)

Vacuum Filter

Millipore Filter 0.45μm- 9 cm diameter Z29078-5 (Sigma)

Oven

Trizma buffer PRE-SET Crystals Type 7.4-FT (Sigma)

Sodium dihydrogen phosphate monohydrate $NaH_2PO_4 \cdot H_2O$ (MERCK)

Sodium Bicarbonate $NaHCO_3$ (ICN)

L-Aspartic Acid monosodium salt (Sigma)

Calcium Chloride Dihydrate $CaCl_2 \cdot 2H_2O$ (MERCK)

DDW/TDW, Filtered.

1) Two solutions were prepared:

Solution I ($CaCl_2$ + Trizma + polymer): 20.0 gr. Trizma, 2.94 gr. $CaCl_2$, polymer, 2.0 liters of DDW.

Solution II (NaH_2PO_4 + Trizma): 20.0 gr. Trizma, 1.66 gr $NaH_2PO_4 \cdot H_2O$, 0.6 gr. $NaHCO_3$ 0.1 gr. L-Aspartic Acid, 2.0 liters of DDW.

2) Equal volumes of solution I and solution II (1.5 liters each) were mixed rapidly in a 4 liter glass bowl. The final solution had the following concentrations: 0.01M $CaCl_2$, 0.006M NaH_2PO_4, 150 ppm (mg/Liter) $NaHCO_3$, 25 ppm L-Aspartic Acid with varying concentration of polymer.

3) The solution was heated in a microwave oven at maximum power for 30 min.

4) The irradiated mixture was placed in an ice bath for 1 hr.

5) The mixture was filtered through a Millipore filter (0.45μm). .

6) The precipitate was washed with 50 ml of DDW.

7) The precipitate was transferred to a glass beaker and dried overnight at 55°-60°C.

8) The dried precipitate was ground to a fine powder using a mortar and pestle.

9) The resulting powder was weighed for quantity determination.

10) The powder was sterilized by γ irradiation.

11) The powder was stored in a closed sterile glass vial and labeled according to date, batch number and quantity.

The polymers were added in the in the amount presented in table 1. The quantities listed do not represent the final concentration

TABLE 1

Additive	Parts per million (ppm)									
Heparin 15 KDa	100	50	5		0.5					
Heparin 6 KDa	100	50	5	1	0.5	0.25	0.1	0.05	0.001	0.0001
SOS		1.66	0.166		0.0166					
Dex Sulphate					0.5					

Heparin -MW 15 kDa; heparin-MW 6 kDa; sucrose octasulfate (SOS) MW-1.16 kDa; dextran sulfate MW 10 kDa. The composites are tested in adsorption, resorption and release assays, in X-ray diffraction and FITR.

Example 2: X-Ray Diffraction analysis

Bone substitute material prepared according to US Patent 6,231,607 and composite prepared according to Example 1 and sterilized by heat sterilization or γ-irradiation was exposed to X-ray. An X-ray diffraction (XRD) pattern was obtained from a packed powder sample of the material pulverized in a mortar and pestle. X-rays were performed using a

using an X-ray powder diffractometer, Rigaku, Japan. The scan rate was set to 0.5 degree/minute over the 2 theta (2θ) angular range from 20°-35°. The step size was set to 0.05°. Figure 1A shows the X-ray diffraction pattern of the bone substitute powder prepared according to US patent 6,231,607. Note the large undifferentiated peak at 2θ=31°-33°. The X-ray diffraction pattern of a composite comprising heparin, wherein the heparin was added to 0.5 parts per million (ppm) is presented in figure 1B. The X-ray diffraction pattern of a composite comprising dextran sulfate added to 0.5 ppm is shown in Figure 1C. The X-ray diffraction patterns of composites comprising sucrose octasulfate added to 0.5 ppm or 5 ppm are shown in Figures 1D and 1E, respectively. In all the cases the X-ray diffraction patterns of the composites show strong similarity to the unradiated material, specifically a characteristic undifferentiated peak at 2 theta 2θ=31°-33°.

Example 3: Scanning Electron Microscope Analysis

Bone substitute composites prepared with various amounts of different polysaccharides was subject to Scanning Electron Microscope Analysis (SEM). Figures 2B and 2C show the aggregate formation of the synthetic apatite-heparin composite (0.5ug/ml and 100ug/ml, respectively) as compared to the synthetic apatite (PCA) alone shown in Figure 2A. The composite prepared from the liquid mixture comprising 0.5 % W/W Heparin MW 6,000 exhibited a distinct structure as seen by SEM. All figures are 39570X magnification.

Example 4: Bone Substitute Composite with a poloxamer copolymer

PLURONIC® is a block co-polymer that may serve to impart select physical and biological properties to the composite and may serve as an FGF stabilizer. The formation of this system is carried out basically using the same steps as for the polymers exemplified above.

The bone substitute composite was prepared using various concentrations of PLURONIC® poloxamer F127 (0.1 ppm-500 ppm).

Example 5: Resorbability Assay

Resorbability of the composite is attributable in part to its crystallinity and chemical composition. Several assays, both in vivo and in vitro are known in the art to analyze resorbability of implantable composites. A solution of EDTA was shown to dissolve the synthetic apatite. An in vitro assay, presented in Example 9, comprises the addition of EDTA to the bone substitute composite, and testing the time it takes for the composite to dissolve.

In vivo assays include intramuscular, subcutaneous and intraosseous models. An in vivo assay measuring subcutaneous resorption of dense carbonate apatite is disclosed in Barralet (Barralet, J. et al., J Biomed Mater Res 49(2):176-82, 2000). Another example discloses the resorption of porous ceramic implants in a dog model (Pollick, S., et al., J Oral Maxillofac Surg, 53(8):915-22; 1995).

Example 6: Injectable Composition

These examples demonstrate the methods of preparing a fluid composition for use as a bone substitute material.

An amount of 0.3 gm dry sterile powder of Step 1 was mixed with 2ml sterile PBS. The composition was mixed for 1 hour on a shaker and filtered through a 0.45 μm membrane to remove excess liquid. Remaining on the filter was approximately 0.5 ml of a pasty substance that was placed into a syringe for local administration in an animal model, as in example 10 below.

An amount of 3 gm dry sterile powder of Step 1 was mixed with 3 ml sterile PBS to yield approximately 5 ml of a pasty substance that was placed into a syringe for local administration in an animal model.

Additional compositions are prepared by varying the w/w or w/v ratio of the composite and a pharmaceutically acceptable diluent. Viscosity of the fluid or semi-fluid compositions was determined by standard techniques.

Example 7: FGFR-Transfected FDCP Proliferation Assay

This assay was used to determine the release of FGF from the composite comprising the synthetic apatite and a polymer, specifically heparin, SOS or dextran sulfate.

The FDCP cell line is a murine immortalized, interleukin 3 (IL-3)-dependent cell line of myelocytic bone marrow origin that does not express endogenous FGF Receptors (FGFR). Upon transfection with FGFR cDNA, the FDCP cell line exhibited a dose-dependent proliferative response to FGF that replaces the dependence on IL-3. FGFR transfected FDCP cells can therefore be used to screen for FGFR signaling. For example, the FDCP cell line stably transfected with FGFR1 responds well to FGF2 and select variants of FGF2 whereas the FDCP cell line stably transfected with FGFR3 responds well to FGF9 and select variants of FGF9. The cells response to various ligands is quantitated by a cell proliferation assay with XTT reagent (Cell Proliferation Kit, Biological Industries Co.). The method is based on the capability of mitochondrial enzymes to reduce

tetrazolium salts into a colorigenic compound, which can be quantitated and is indicative of cell viability.

Specifically, FDCP cells stably expressing the FGFR1 (FDCP-FGFR1) were grown in "full medium" (Iscove's Medium containing 2ml glutamine, 10% FCS, 100ug/ml penicillin, 100ug/ml streptomycin) supplemented with 5ug/ml heparin. Cells were split every 3 days and kept in culture no more than one month. One day prior to the experiment the cells were split. Before the experiment the cells were washed 3 times (1000 rpm, 6 min) with full medium. The cells were resuspended and counted with Trypan Blue. Twenty thousand (2 x 10^4) cells were added to each well of 96-well plate in 50 µl full medium containing heparin. Condition medium containing FGF or FGF complexed with the various polysaccharides was added in an additional volume of 50 µl full medium to bring the final volume to 100 µl. The plate was incubated for 48 hours at 37°C. To test cell proliferation, 100 µl of PMS reagent was added to 5 ml of XTT reagent and mixed well (according to manufacture protocol). 50 µl of the latter solution were aliquoted into each well, and the plates incubated at 37°C for 4 hours and the color developed was read by a spectro-ELISA reader at A_{490nm}.

Example 8: Release of FGF from the Composite

FGF was used as the bioactive agent and both the synthetic apatite and synthetic apatite-heparin composite were tested as carriers.

An FGF (FGF2 variant, 200 µl of a 1, 10 or 20 um/ml solution) was added to 50 µl of synthetic apatite and synthetic apatite -heparin composite and allowed to adsorb for 1 hour at room temperature (RT). The synthetic apatites were centrifuged, the supernatant removed, and washed with 1x PBS. Two independent assays were performed on this material. An ELISA, as described in example 9, was carried out to establish the amount of FGF that bound to the synthetic apatite and synthetic apatite-heparin composite. An FDCP proliferation assay, as described in example 7, was carried out to determine whether the FGF2 variant that bound to the material remained active.

Figure 3A shows the amount of FGF that was able to bind to either the synthetic apatite prepared according to US Patent 6,231,607 (PCA) and the various synthetic apatite-polymer composites as determined by direct binding ELISA.

Figure 3B shows the results of the proliferation assay of FGFR1 expressing FDCP cells in the presence of composite comprising FGF and suggests that the amount of FGF released

and the rate of release from the composite depended on the type of polymer incorporated into the composite. SOS refers to the synthetic apatite-polymer composite comprising SOS (0.5 ppm added ab initio). A majority of the FGF that bound was released within the first day. "hep" refers to the synthetic apatite-heparin composite comprising heparin (MW 6,00; 0.5 ppm). A large proportion of the FGF added to the composite was adsorbed by the composite (85%) and the amount of FGF released was spread out over the 7 weeks of the assay. This results suggests that the synthetic apatite-heparin composite provides a good delivery system for controlled release of FGF. It will be apparent to a person with skill in the art that different polysaccharides, preferably glycosaminoglycans including heparin derivatives, including very low molecular weight heparin, low molecular weight heparin, heparin derivatives and heparin mimetics may be used in place of the heparin tested herein to control release of specific growth factors to different tissue types. The PCA showed reduced release of FGF over time, despite the fact that 20% of the FGF added was adsorbed to the material. The dextran adsorbed FGF quite poorly (>5%) and it appears that the FGF was released in a regular manner, albeit in small doses.

The rate of release of a bioactive agent the composite may be optimized for different applications and tissue types. For example, endochondral bone formation may benefit from a different release pattern of FGFs that intermembranous bone formation. In infected tissue, fast release of an antimicrobial and slow release of a growth factor may enhance healing.

Example 9: Direct binding ELISA

This assay was performed to quantify the binding of a bioactive agent to the poorly crystalline apatite or to the synthetic apatite and a polymer composite.

For the ELISA assay the 50 ul of sample was dissolved in 1 ml of 250 mM EDTA for 5-7 hours at RT with shaking and measured as follows:

The wells of the plate were coated with an FGF/sodium bicarbonate solution. A calibration curve was prepared for FGF concentrations in the range of 250ng/ml to 8ng/ml. Dilutions of test-samples (PCA or PCA-derivative adsorbed with a bioactive agent) were prepared in bicarbonate-buffer to get a final concentration of 0.1 M bicarbonate. The dilutions of the test samples were in a range so that the predicted concentration of FGF fell in the linear area of the calibration.

To each well of a 96 well plate (NUNC immunoplate, F96 maxisorp) 100 µl of each test sample was added. The plate was covered with Parafilm™ and incubated at 4°C overnight (ON). The wells were washed with 2M NaCl once and with PBS twice. Detection was carried out as follows: the wells were blocked with 2% BSA by adding 300 µl 2% BSA to each well. The samples were incubated for 1 hour at room temperature (R.), or at 4°C ON. The wells were washed five times with 300 µl PBST (0.5 ml Tween-20 to 500 ml PBS). The antibody, 100 µl of DG2 (anti FGF2 1:5,000) was added to each well except control. The samples were incubated 2 hours at RT and washed thrice with PBST. One hundred microliter (100µl) secondary antibody (HRP-conjugated Goat α mouse 1:10,000) was added to each well followed by 3 washes with PBST. TMB substrate (100 µl) was added to each well and the samples incubated at RT until the desired color developed, after about 10 min. and the reaction stopped by adding 50 µl 1M H_2SO_4 to each well. The plate was read in an ELISA spectrophotometer at $A_{450\ nm}$.

Example 10: Rat Tibia Model

Objectives: To investigate the histological differences in bone ingrowth using injectable bone graft composition of the invention compared to commercially available bone graft composition.

The following tables show the experimental setup of 5 trials.

1) Table 2 shows the experimental setup for an 8 week animal experiment wherein the defect was a hole in the tibial bone. PCA was compared to PCA-heparin composite and a PCA heparin composite further comprising an FGF2 variant.

Table 2

Rat no.	tag	LT leg treatment	RT leg treatment
1	No tag	PCA	No treatment
2	Tail cut	PCA+Heparin	No treatment
3	Tail cut	PCA+Heparin	No treatment
4	RT ear cut	PCA+Heparin+FGF2v	HA+Heparin+FGF2v
5	LT ear cut	PCA+ FGF2v	PCA+ FGF2v

2) Table 3 shows the setup of an 8 week experiment with animals having a wedge defect. The effect of PCA alone was compared to the effect of PCA-SOS composite and a PCA-SOS composite (SOS, 0.5 ppm) further comprising an FGF2 variant (FGF2v).

Table 3

No.	tag	LT leg	RT leg
1	No tag	No treatment	No treatment
2	Tail cut	PCA	PCA
3	RT ear cut	PCA + SOS	PCA +SOS
4	∇ in ear	PCA + FGF2v	PCA + FGF2v
5	∇ in ear	PCA + FGF2v	PCA + FGF2v
6	Lt ear cut	PCA + SOS + FGF2v	PCA + SOS + FGF2v
7	Lt ear cut	PCA + SOS + FGF2v	PCA + SOS + FGF2v

Figure 4 A shows the filling in of the wedge defect with new bone surrounding the PCA particles. Figure 4B shows the bone repair surrounding the PCA-SOS aggregates. Figure 4B shows the presence of more PCA particles remaining in the wedge suggesting that the Composite has better cohesive properties than the PCA alone.

3) Experiment 3, Table 4, was designed to test the effect of FGF2v , FGF9v and combination of both growth factors in a synthetic apatite alone in a wedge model.

Table 4

#	Tag	Lt leg	Rt leg
1	No tag	PCA	PCA
2	Tail cut	PCA + 2.5μg FGF2V	PCA + 2.5μg FGF2V
3	Tail cut	PCA + 2.5μg FGF2V	PCA + 2.5μg FGF2V
4	Tail cut	PCA + 2.5μg FGF2V	PCA + 2.5μg FGF2V
5	LT ear cut	PCA + 2.5μg FGF9V	PCA + 2.5μg FGF9V
6	LT ear cut	PCA + 2.5μg FGF9V	PCA + 2.5μg FGF9V
7	LT ear cut	PCA + 2.5μg FGF9V	PCA + 2.5μg FGF9V
8	RT ear cut	PCA + 2.5μg FGF9V+ 2.5μg FGF2V	PCA + 2.5μg FGF9V+ 2.5μg FGF2V
9	RT ear cut	PCA + 2.5μg FGF9V+ 2.5μg FGF2V	PCA + 2.5μg FGF9V+ 2.5μg FGF2V

Figures 5 A and B show histological samples of the bone filling at x100 in a sample wherein a paste of PCA and 2.5 ug FGF2v was added. Note the formation of good quality bone and the infiltration of blood vessels (BV) capable of delivering bone forming cells to the site. This surprising result shows that the PCA carrying a growth factor to the site of a defect results in bone repair and the formation of blood vessels into the site of the defect.

3) Experiment 4, Table 5, was designed to test the effect of different concentrations of FGF2v and FGF9v on healing, in a synthetic apatite-heparin composite in a wedge model.

Table 5

No.	Tag	Lt leg	Rt leg
1	No tag	PCA + HEP	PCA + HEP
2	No tag	PCA + HEP	PCA + HEP
3	Tail cut	PCA + HEP + 0.04μg FGF2V	PCA + HEP + 0.04μg FGF2V
4	Tail cut	PCA + HEP + 0.04μg FGF2V	PCA + HEP + 0.04μg FGF2V
5	LT ear cut	PCA + HEP + 0.2μg FGF2V	PCA + HEP + 0.2μg FGF2V
6	LT ear cut	PCA + HEP + 0.2μg FGF2V	PCA + HEP + 0.2μg FGF2V
7	RT ear cut	PCA + HEP + 1μg FGF2V	PCA + HEP + 1μg FGF2V
8	RT ear cut	PCA + HEP + 1μg FGF2V	PCA + HEP + 1μg FGF2V
9	2 ears	PCA + HEP + 0.2μg FGF9V	PCA + HEP + 0.2μg FGF9V
10	2 ears	PCA + HEP + 0.2μg FGF9V	PCA + HEP + 0.2μg FGF9V
11	No tag	PCA + HEP	PCA + HEP
12	No tag	PCA + HEP	PCA + HEP
13	Tail cut	PCA + HEP + 0.04μg FGF2V	PCA + HEP + 0.04μg FGF2V
14	Tail cut	PCA + HEP + 0.04μg FGF2V	PCA + HEP + 0.04μg FGF2V
15	LT ear cut	PCA + HEP + 0.2μg FGF2V	PCA + HEP + 0.2μg FGF2V
16	LT ear cut	PCA + HEP + 0.2μg FGF2V	PCA + HEP + 0.2μg FGF2V
17	RT ear cut	PCA + HEP + 1μg FGF2V	PCA + HEP + 1μg FGF2V
18	RT ear cut	PCA + HEP + 1μg FGF2V	PCA + HEP + 1μg FGF2V
19	2 ears	PCA + HEP + 0.2μg FGF9V	PCA + HEP + 0.2μg FGF9V
20	2 ears	PCA + HEP + 0.2μg FGF9V	PCA + HEP + 0.2μg FGF9V

5) The following table shows the experimental setup to compare the synthetic apatite polymer of the invention to a known Calcium Phosphate material. The material is a ceramic crystalline hydroxyapatite, having high aggregate size of approximately 1 μm. Holes were made in the rat tibiae.

No.	Tag	Left leg	Right leg
1	No tag	PCA + HEP + Gelatin	PCA + HEP + Gelatin
2	No tag	PCA + HEP + Gelatin	PCA + HEP+ Gelatin
3	Tail cut	PCA + HEP + Gelatin + FGF9V	PCA + HEP + Gelatin + FGF9V
4	Tail cut	PCA + HEP + Gelatin + FGF9V	PCA + HEP + Gelatin + FGF9V
5	RT ear cut	PCA + HEP + Gelatin + FGF2V	PCA + HEP + Gelatin + FGF2V
6	RT ear cut	PCA + HEP + Gelatin + FGF2V	PCA + HEP + Gelatin + FGF2V
7	LT ear cut	Competitor $CaPO_4$	Competitor $CaPO_4$
8	LT ear cut	Competitor $CaPO_4$	Competitor $CaPO_4$
9	both ears	Competitor $CaPO_4$ + Gelatin +FGF2V	Competitor $CaPO_4$ + Gelatin +FGF2V
10	both ears	Competitor $CaPO_4$ + Gelatin +FGF2V	Competitor $CaPO_4$ + Gelatin +FGF2V
11	both ears	Competitor $CaPO_4$ + Gelatin +FGF2V	Competitor $CaPO_4$ + Gelatin +FGF2V
12	No tag	PCA + HEP + Gelatin	PCA + HEP + Gelatin
13	No tag	PCA + HEP + Gelatin	PCA + HEP + Gelatin
14	Tail cut	PCA + HEP + Gelatin + FGF9V	PCA + HEP + Gelatin + FGF9V
15	Tail cut	PCA + HEP + Gelatin + FGF9V	PCA + HEP + Gelatin + FGF9V
16	RT ear cut	PCA + HEP + Gelatin + FGF2V	PCA + HEP + Gelatin + FGF2V
17	RT ear cut	PCA + HEP + Gelatin + FGF2V	PCA + HEP + Gelatin + FGF2V
18	LT ear cut	Competitor $CaPO_4$	Competitor $CaPO_4$
19	LT ear cut	Competitor $CaPO_4$	Competitor $CaPO_4$
20	both ears	Competitor $CaPO_4$ + Gelatin +FGF2V	Competitor $CaPO_4$ + Gelatin +FGF2V

Surgical procedure: Animals were anesthetized according to a standard protocol, using intramuscular (IM) injection of 85mg/kg ketamine and 3mg/kg xylazine.

A defect was created in the proximal tibial metaphysis, 3-4 mm below the collateral ligament insertion, by drilling a hole of 2 mm diameter and 2-3 mm in depth or by cutting a wedge of approximately 1.5 mm deep and 3 mm wide.

The defect was treated according to the aforementioned table by locally administering 0.02ml poorly crystalline apatite of the invention using a 1 ml syringe.

Quality evaluation: at the end of 7-8weeks rats were sacrificed and the defect area evaluated histologically for gross cell morphology, cell abundance and the appearance of extra-cellular material.

Standard histological staining methods were used (H&E) and the tissue samples were scored by a pathologist for evaluation of histological changes during the healing process.

Example 11: Rat Calvarial Model

Two rat calvarial defect models are used to determine the efficacy of the composition of the invention to induce bone repair of large defects. In one model, two 3mm defects per calvaria are drilled using a trephine on both sides of the median suture; one side serves as a control. The protocol and evaluation method is described in Colombier (Colombier et al., Cells Tissues Organs 164:131-140, 1999).

The second model, described in Hollinger (Hollinger and Kleinschmidt, J Craniofacial Surg 1:60-68, 1990) introduces an 8 mm defect in the parietal bone. The defect is filled with the composition of the invention and the incision site sutured. Following 4 weeks the animals are euthanized and the defect sites recovered. Histological analysis proceeds as in example 12.

Example 12: Non-Union Model

Distraction osteogenesis is a useful method for bone elongation of extremities in short stature, for example in individuals diagnosed with different forms of dwarfism such as achondroplasia. This process of bone lengthening is long and often complications arise such as non-union or poor quality of the regenerated bone.

The maximal rate of elongation used in the current procedure of limb elongation, while maintaining proper bone healing and reconstitution is approximately 1mm/day. Faster elongation rates have resulted in lack of fusion or in the formation of weak bone that

breaks easily or is not weight bearing. For this reason, and in order to enable a shorter elongation period with the concomitant formation of strong, healthy bone it is necessary to provide graft substitute that will promote bone regeneration. For optimal comparison to humans, it is important to perform the procedure in a large animal model like sheep, preferably with analogous devices and elongation techniques. The model is adjusted to test enhancing bone formation at an elongation rate of 1.5mm/day.

Materials and Methods

Treatment arms:

Treatment 1: 6 lambs (6 limbs), Control no treatment

Treatment 2: 6 lambs (6 limbs), fluid bone substitute of the invention

Treatment 3: 6 lambs (6 limbs): Heparin modified synthetic apatite

Treatment 4: 6 lambs (6 limbs): Heparin modified synthetic apatite with FGF2 variant

Lambs are assigned randomly into one of the two treatment arms. Surgical lengthening of the right femur is performed in 24 sheep aged from 3 to 4 months.

Anesthesia and pre-mediation: General anesthesia is given without endotracheal intubation. Intramuscular atropine is given as premedication (0.5 mg/kg), and thiopentone sodium-2.5% (10-15 mg/kg), Fentanyl® (0.0015 mg/kg) and Diazepam® (0.2 mg/kg) is administered intravenously.

Fixation: A monolateral external fixator (Monotube-Triax®, Stryker Trauma, Geneva, Switzerland) with four pins, two proximal and two distal in each of its pin clamps, are positioned so that the pins are kept away from the growth plates and the surface of the joint. The osteotomy is performed using a pneumatic saw.

Lengthening: The lengthening procedure begins seven days after surgery for all treatment groups and continues until the limb is lengthened by 5 cm. The total elongation period lasts 33 days, at a rate of 1.5 mm/day.

Treatment 1 - Control - To assess the effect during the consolidation period, animals undergo surgery but receive no treatment.

Treatment 2 - To assess the effect of the bone substitute, the bone substitute is administered once, one week after completion of elongation.

Treatment 3 - To assess the effect of the carrier alone during consolidation period heparin modified synthetic apatite will be administered once, one week after completion of elongation.

Treatment 4 - To assess the effect of the product during consolidation period, heparin modified synthetic apatite with FGF2 variant will be administered once, one week after completion of elongation.

Animals are kept in a limited area, during the extent of the whole experiment and will be allowed to feed and walking ad libitum in cage. Animals are weighed at fixed intervals and general well-being is monitored.

To study bone formation in the host bone, four different bone markers fluorochromes are administered IM, according to the following schedule: one week after surgery: calcein (green, Sigma®); two weeks after surgery: alizarin (red, Sigma®); three weeks after surgery: xylenol (orange, Fluka®) and three days before sacrifice oxytetracycline (yellow, Duphacycline®).

Assessment of efficacy: Success is determined by healing and bone quality obtained after elongation.

X-ray: Progress of bone healing will be controlled by X-ray at weeks 1, 2 and 4 after beginning of elongation. The parameters to be assessed from the X-ray are: degree of callus formation, gap closure and remodeling achieved during treatment. X-ray scoring is performed by an orthopedic surgeon, according to established bone healing grading systems.

The Spalteholz technique to analyze the vascularization of the lengthened callus in each group is performed after intraarterial injection of Berlin blue through the femoral artery before sacrifice.

Completion: The animals are sacrificed three months after initial surgery by IV injection of 5 meq of KCl, after anesthesia with sodium pentobarbital (1.5 mg/kg weight).

Histology: The callus is divided into two parts, one for embedding in paraffin, and the other undecalcified, for embedding in methylmethacrylate.

For the histological study, the specimens are fixed in Bouin for 24 hours and decalcified in a solution of PVP-EDTA, at 4° C. Once specimens are decalcified, they are dehydrated using alcohols of increasing proof (70%, 80%, 96% and 100%), and after 4 hours in

xylene, they are embedded in paraffin at a temperature of 60°C. The specimens are sectioned at 4 μm, and stained with Masson's trichrome, hematoxylin and eosin (H&E), safranin-O and von Kossa.

To analyze the mineralization by fluorochromes, the specimens are fixed in formalin for one week, then dehydrated using alcohols of increasing proof. After one week in PMMA-alcohol and three weeks in PMMA (Technovit 7200 VLC®), specimens are sectioned with a diamond saw (Exakt®) and trimmed to a thickness of 14 μm. After measuring the sections with ultraviolet light the distance of the bone markers is measured and the bone index formation calculated (distance mm/days)

The proximal parts of control and test tibiae are extracted and cut in lateral and medial parts. The lateral portion is placed in 4% buffered formaldehyde. After decalcification in EDTA, the specimens are embedded in paraffin and cut into 4 μm slices. The H&E, Masson's trichrome, Safranin-0 and Alcian blue-PAS stains are applied according to standard technique.

Immunohistochemistry: Antibodies to collagen I, collagen II, FGF1, and S-100 are used to detect protein expression in the lengthened callus by an indirect two-step method: 4 μm paraffin sections are trypsinized and deparaffinized. Endogenous peroxidase is blocked by placing the sections in hydrogen peroxidase solution for 30 min. They are incubated in the following reagents with appropriate Tris-buffered-saline (TBS: 0.55 M, pH 7.36) washes: normal pig serum for 30 min, primary antibody for 1 hour, secondary biotinylated antibody for 30 min, and avidin-biotin complex (Dako KO355) for 30 min. The reactions are visualized with chromogen substrate solution (diaminobenzidine, hydrogen peroxidase, TB) and sections are counterstained with Harris's hematoxylin, dehydrated, and mounted. As a negative control, TBS is used instead of the primary antibodies. All stained sections are examined and photographed with use of a microscope (Nikon Optiphot-2®, Japan).

Morphometric analysis: An image analyzing system (Leica Q 500 MC ®) determines the histomorphometric parameters. The parameters determined using Masson's trichrome stain are: Trabecular width; Trabecular area; Trabecular erosion surface; Index of trabecular erosion; Number of osteoblasts; Number of osteoclasts per field; Number of osteoclast nuclei; Index of bone reabsorption or number osteoclast nuclei / osteoclasts.

The parameters determined using von Kossa's stain are: Osteoid width; Osteoid-trabecular index; and the fluorescence staining measures the extent of long bone formation.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying knowledge within the skill of the art, including the contents of the references cited herein, readily modify and/or adapt for various applications such specific embodiments, without undue experimentation, without departing from the general concept of the present invention. Therefore, such adaptations and modifications are intended to be within the meaning and range of equivalents of the disclosed embodiments, based on the teaching and guidance presented herein. It is to be understood that the phraseology or terminology herein is for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and guidance presented herein, in combination with the knowledge of one of ordinary skill in the art.

Claims

1. A bone substitute composite comprising a synthetic apatite, at least one amino acid in monomeric or polymeric form, carbonate, at least one polymer, optionally further comprising a bioactive agent.

2. The bone substitute composite according to claim 1 wherein said at least one polymer is selected from the group consisting of natural polymers or synthetic polymers.

3. The bone substitute composite according to claim 2 wherein said natural polymer is a polysaccharide.

4. The bone substitute composite according to claim 3 wherein said polysaccharide is a glycosaminoglycan.

5. The bone substitute composite according to claim 4 wherein said glycosaminoglycan is heparin, a heparin derivative or a heparin mimetic.

6. The bone substitute composite according to claim 1 wherein said at least one bioactive agent is selected from the group consisting of antibiotics, antiviral agents, chemotherapeutic agents, anti-rejection agents, analgesics and analgesic combinations, anti-inflammatory agents, hormones, growth factors and cytokines.

7. The bone substitute composite according to claim 6 wherein said at least one bioactive agent is a growth factor.

8. The bone substitute composite according to claim 7 wherein said growth factor is a fibroblast growth factor or an active fragment or variant thereof.

9. The bone substitute composite according to claim 1 wherein said synthetic apatite is a poorly crystalline apatite.

10. The bone substitute composite according to claim 1 wherein said synthetic apatite is a poorly crystalline apatite and said polymer is heparin, heparin derivative or a heparin mimetic.

11. The bone substitute composite according to claim 10 further comprising a at least one bioactive agent

12. The bone substitute composite according to claim 11 wherein said at least one bioactive agent is a growth factor.

13. The bone substitute composite according to claim 12 wherein said growth factor is a fibroblast growth factor or an active fragment or variant thereof.

14. The bone substitute composite according to claim 9 wherein said poorly crystalline apatite has an X-ray diffraction pattern comprising a broad peak at 2 theta values of about 31°-33°.

15. A pharmaceutical composition comprising a composite comprising a synthetic apatite, at least one amino acid in monomeric or polymeric form, carbonate, at least one additional polymer optionally further comprising at least one bioactive agent and a pharmaceutically acceptable carrier or diluent

16. The pharmaceutical composition according to claim 14 wherein said at least one polymer is selected from the group consisting of natural polymers or synthetic polymers.

17. The pharmaceutical composition according to claim 15 wherein said natural polymer is a polysaccharide.

18. The pharmaceutical composition according to claim 16 wherein said polysaccharide is a glycosaminoglycan.

19. The pharmaceutical composition according to claim 17 wherein said glycosaminoglycan is heparin, a heparin derivative or a heparin mimetic.

20. The pharmaceutical composition according to claim 14 wherein said the at least one bioactive agent is selected from the group consisting of antibiotics, antiviral agents, chemotherapeutic agents, anti-rejection agents, analgesics and analgesic combinations, anti-inflammatory agents, hormones, growth factors and cytokines.

21. The pharmaceutical composition according to claim 19 wherein said at least one bioactive agent is a growth factor.

22. The pharmaceutical composition according to claim 20 wherein said growth factor is a fibroblast growth factor or an active fragment or variant thereof.

23. The pharmaceutical composition according to claim 14 wherein said synthetic apatite is a poorly crystalline apatite.

24. The pharmaceutical composition according to claim 22 further comprising heparin, heparin derivative or a heparin mimetic.

25. The pharmaceutical composition according to claim 23 further comprising at least one bioactive agent.

26. The pharmaceutical composition according to claim 25 wherein said at least one bioactive agent is a growth factor.

27. The pharmaceutical composition according to claim 26 wherein said growth factor is a fibroblast growth factor or an active fragment variant thereof.

28. The pharmaceutical composition according to claim 23 wherein said poorly crystalline apatite has an X-ray diffraction pattern comprising a broad peak at 2 theta values of about 31°-33°.

29. A method for treating orthopedic, periodontal and craniofacial indications comprising administering to a subject in need thereof a therapeutically effective amount of a composition comprising a composite comprising synthetic apatite, at least one amino acid in monomeric or polymeric form, carbonate, at least one additional polymer, optionally further comprising at least bioactive agent.

30. The method according to claim 29 wherein said at least one polymer is selected from the group consisting of natural polymers or synthetic polymers.

31. The method according to claim 30 wherein said natural polymer is a polysaccharide.

32. The method according to claim 31 wherein said polysaccharide is a glycosaminoglycan.

33. The method according to claim 32 wherein said glycosaminoglycan is heparin, a heparin derivative or a heparin mimetic.

34. The method according to claim 29 wherein said the at least one bioactive agent is selected from the group consisting of antibiotics, antiviral agents, chemotherapeutic agents, anti-rejection agents, analgesics and analgesic combinations, anti-inflammatory agents, hormones, growth factors and cytokines.

35. The method according to claim 34 wherein said at least one bioactive agent is a growth factor.

36. The method according to claim 35 wherein said growth factor is a fibroblast growth factor or an active fragment or variant thereof.

37. The method according to claim 36 wherein said synthetic apatite is a poorly crystalline apatite.

38. The method according to claim 37 comprising heparin, heparin derivative or a heparin mimetic.

39. The method according to claim 38 further comprising at least one bioactive agent.

40. The method according to claim 39 wherein said at least one bioactive agent is a growth factor.

41. The method according to claim 40 wherein said growth factor is a fibroblast growth factor or active fragment or variant thereof.

42. The method according to claim 37 wherein said poorly crystalline apatite has an X-ray diffraction pattern comprising a broad peak at 2 theta values of about 31°-33°.

43. Use of a composite comprising synthetic apatite, at least one amino acid in monomeric or polymeric form, carbonate, at least one additional polymer and optionally further comprising at least bioactive agent for the manufacture of a medicament for treating diseased or injured bone in orthopedic, periodontal and craniofacial indications.

44. Use according to claim 43 wherein said at least one polymer is selected from the group consisting of natural polymers or synthetic polymers.

45. Use according to claim 2 wherein said natural polymer is a polysaccharide.

46. Use according to claim 34 wherein said polysaccharide is a glycosaminoglycan.

47. Use according to claim 4 wherein said glycosaminoglycan is heparin, a heparin derivative or a heparin mimetic.

48. Use according to claim 1 wherein said at least one bioactive agent is selected from the group consisting of antibiotics, antiviral agents, chemotherapeutic

agents, anti-rejection agents, analgesics and analgesic combinations, anti-inflammatory agents, hormones, growth factors and cytokines.

49. Use according to claim 48 wherein said at least one bioactive agent is a growth factor.

50. Use according to claim 49 wherein said growth factor is a fibroblast growth factor or active fragment or variant thereof.

51. Use according to claim 43 wherein said synthetic apatite is a poorly crystalline apatite.

52. Use according to claim 51 wherein said synthetic apatite is a poorly crystalline apatite and said polymer is heparin, heparin derivative or a heparin mimetic.

53. Use according to claim 52 further comprising a at least one bioactive agent

54. Use according to claim 53 wherein said at least one bioactive agent is a growth factor.

55. Use according to claim 54 wherein said growth factor is a fibroblast growth factor or an active fragment or variant thereof.

56. Use according to claim 51 wherein said poorly crystalline apatite has an X-ray diffraction pattern comprising a broad peak at 2 theta values of about 31°-33°.

57. A method of preparing a bone substitute composite comprising the steps of:

a) preparing a liquid mixture of ionic calcium, phosphate, an amino acid in either monomeric or polymeric form and carbonate, at least one additional polymer, optionally further comprising at least one bioactive agent.

b) subjecting said mixture to microwave irradiation;

c) quenching said irradiated mixture;

d) filtering said quenched mixture so as to separate between the filtrate and a cake;

e) drying said cake;

f) grinding said cake into a powder;

g) sterilizing said powder;

h) wetting said sterilized powder with a solution optionally comprising at least one bioactive agent;

i) preparing said wetted powder for administration.

58. The method according to claim 57 wherein said liquid mixture of step a) comprises calcium chloride, sodium phosphate, L-aspartic acid, sodium carbonate and at least one polymer.

59. The method according to claim 58 wherein said polymer is selected from a group consisting of natural polymers or synthetic polymers.

60. The method according to claim 59 wherein said natural polymer is a polysaccharide.

61. The method according to claim 60 wherein said polysaccharide is a glycosaminoglycan.

62. The method according to claim 61 wherein said glycosaminoglycans is heparin, heparin derivative or a heparin mimetic.

63. The method according to claim 57 sterilizing the powder retains its X-ray diffraction pattern.

64. The method according to claim 63 wherein the sterilization is γ-irradiation.

65. The method according to claim 57 wherein the solution of step h) is a pharmaceutically acceptable fluid.

66. The method according to claim 65 wherein the wetted powder has a paste-like or putty-like consistency.

67. The method according to claim 65 further comprising at least one bioactive agent.

68. The method according to claim 65 wherein said least one bioactive agent is a growth factor.

69. The method according to claim 65 wherein said growth factor is a fibroblast growth factor or an active fragment or variant thereof.

70. A bone substitute composite comprising a synthetic apatite, at least one amino acid in monomeric or polymeric form, carbonate, further comprising at least one bioactive agent.

71. The composite according to claim 70 wherein said at least one bioactive agent is a growth factor

72. The composite according to claim 71 wherein said growth factor is a fibroblast growth factor or an active fragment or variant thereof.

73. A pharmaceutical composition comprising a bone substitute composite comprising a synthetic apatite, at least one amino acid in monomeric or polymeric form, carbonate, further comprising at least one bioactive agent.

74. The pharmaceutical composition according to claim 73 wherein said at least one bioactive agent is a growth factor

75. The pharmaceutical composition according to claim 74 wherein said growth factor is a fibroblast growth factor or an active fragment or variant thereof.

76. A method for treating orthopedic, periodontal and craniofacial indications comprising administering to a subject in need thereof a therapeutically effective amount of a composition comprising a composite comprising synthetic apatite, at least one amino acid in monomeric or polymeric form, carbonate, further comprising at least bioactive agent.

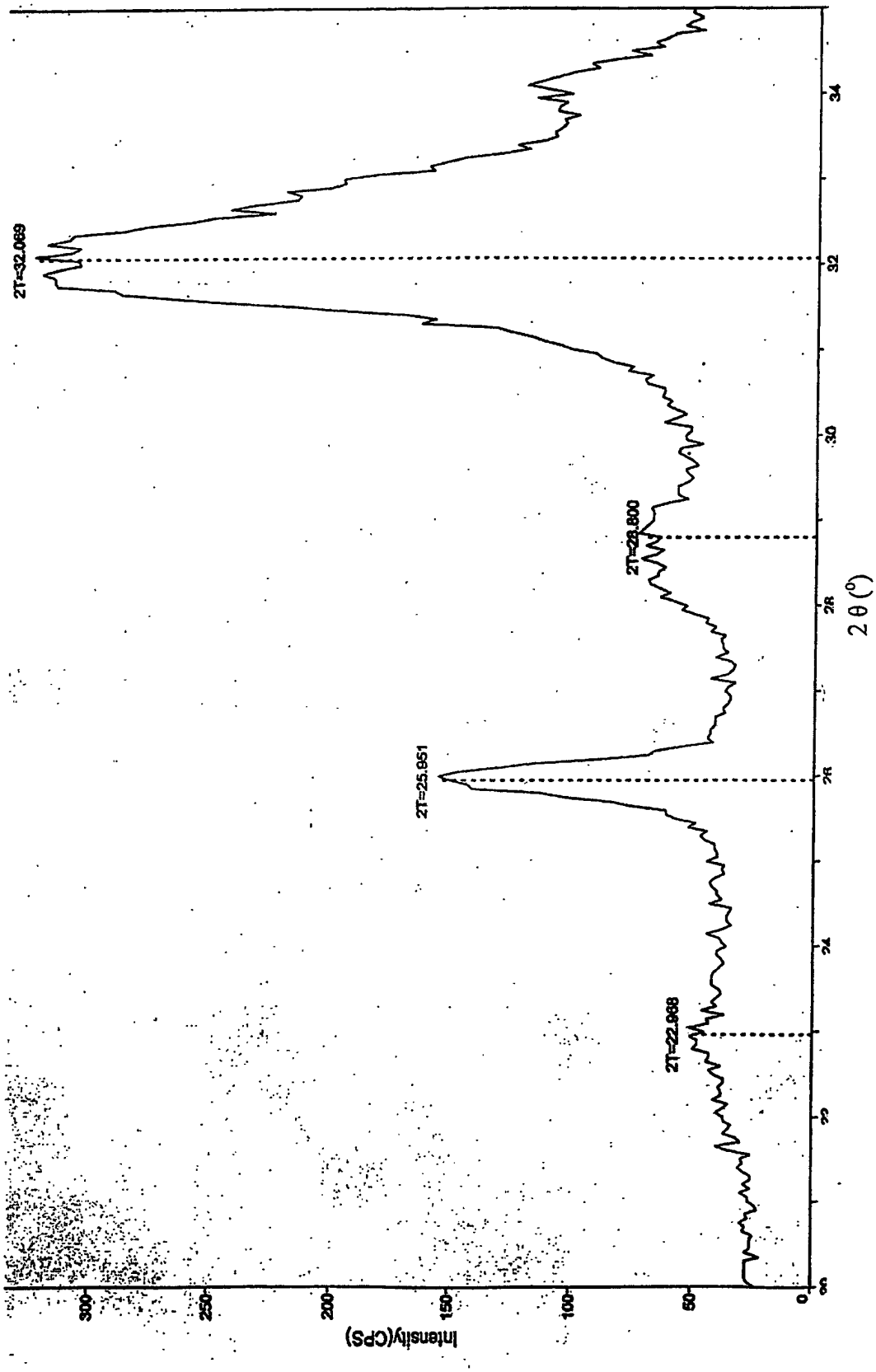
77. The method according to claim 76 wherein said at least one bioactive agent is a growth factor

78. The method according to claim 77 wherein said growth factor is a fibroblast growth factor or an active fragment or variant thereof.

Abstract

A bone substitute composite material comprising a synthetic apatite and at least one polymer useful as a bone graft implant, methods of preparing said composite and uses thereof are provided. The physical and biological properties of the composite are controlled by the addition of certain polymers as well as optional bioactive agents. The composite may be used as a powder, a paste or an implant.

Figure 1A



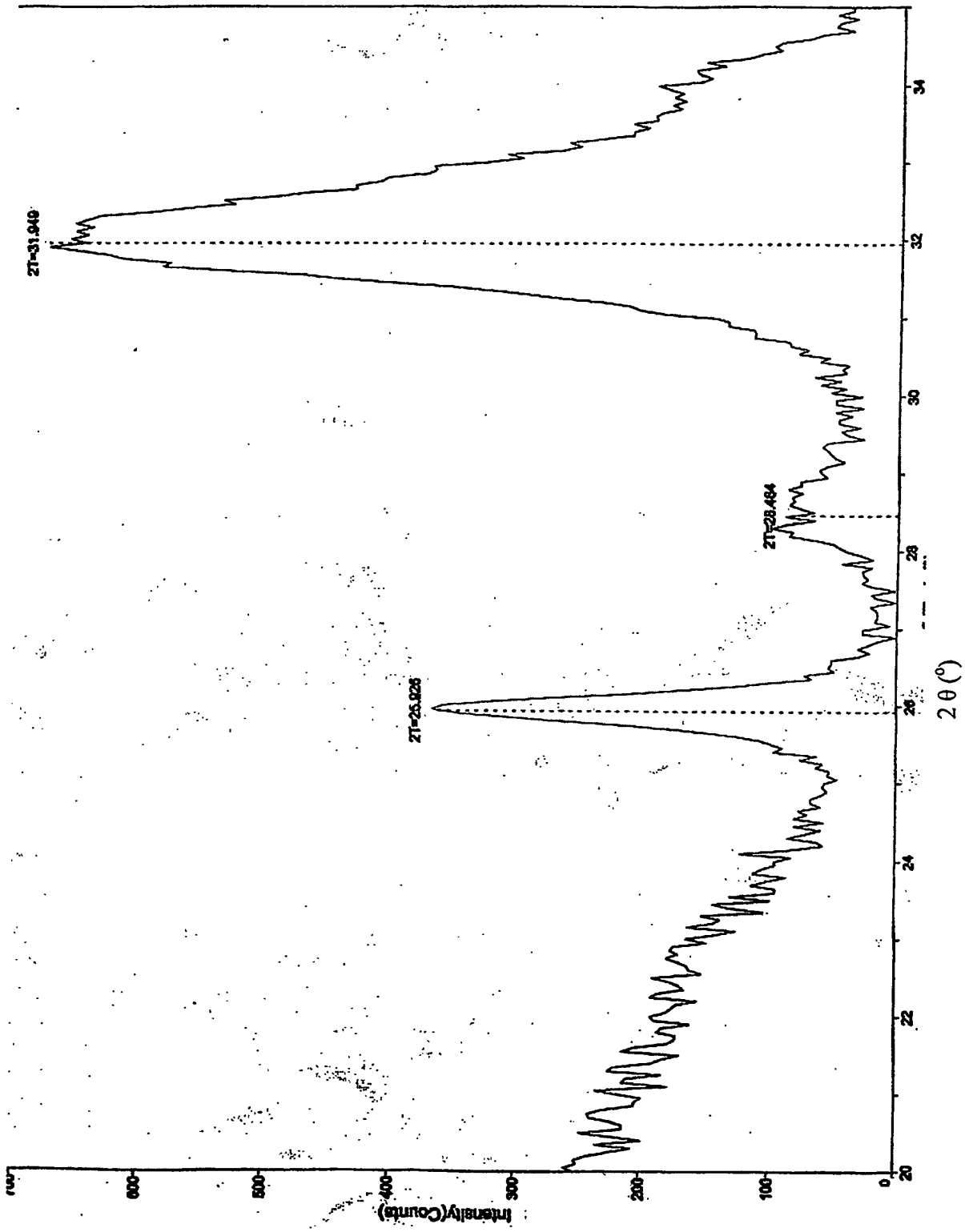


Figure 1B

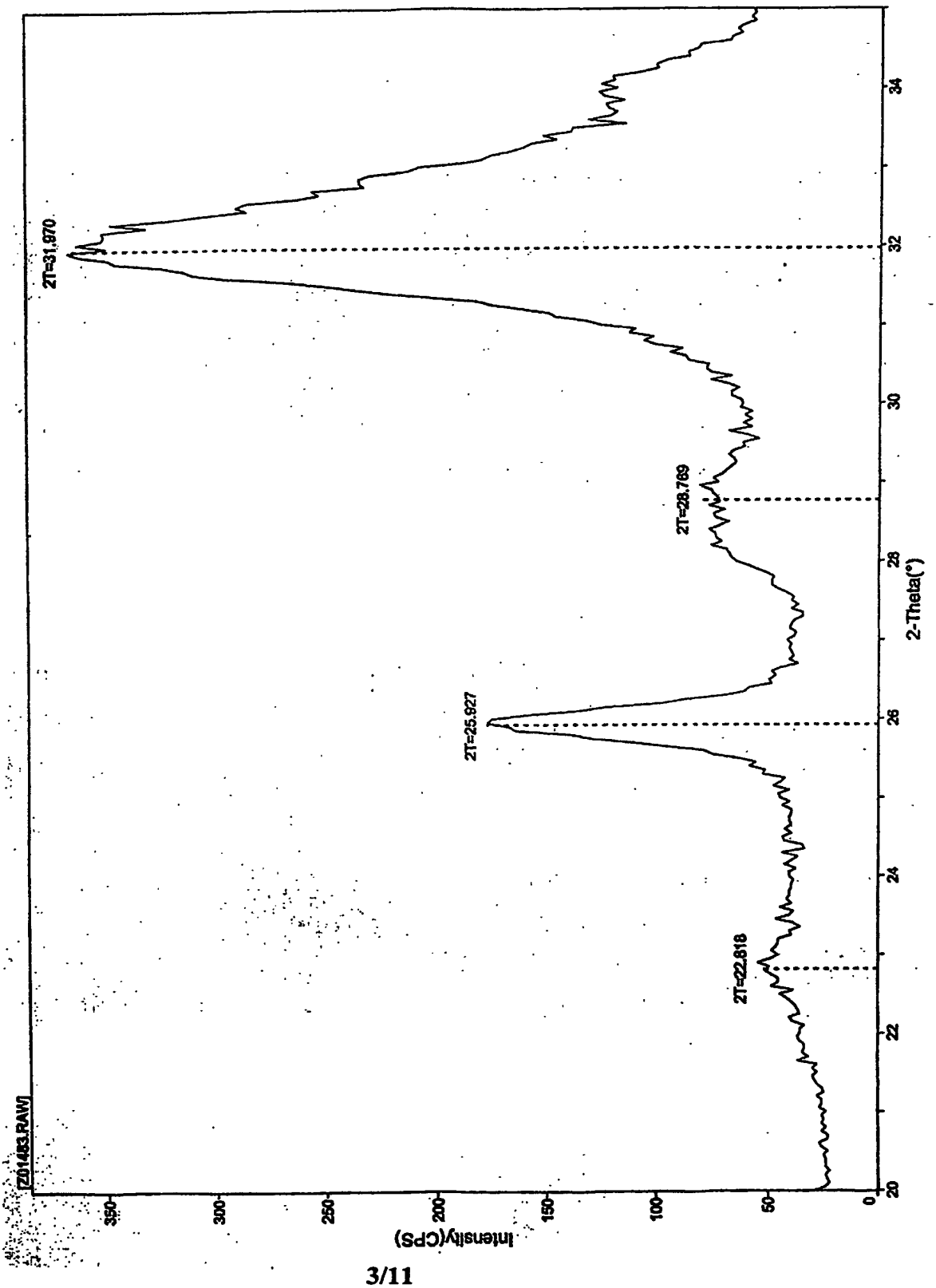

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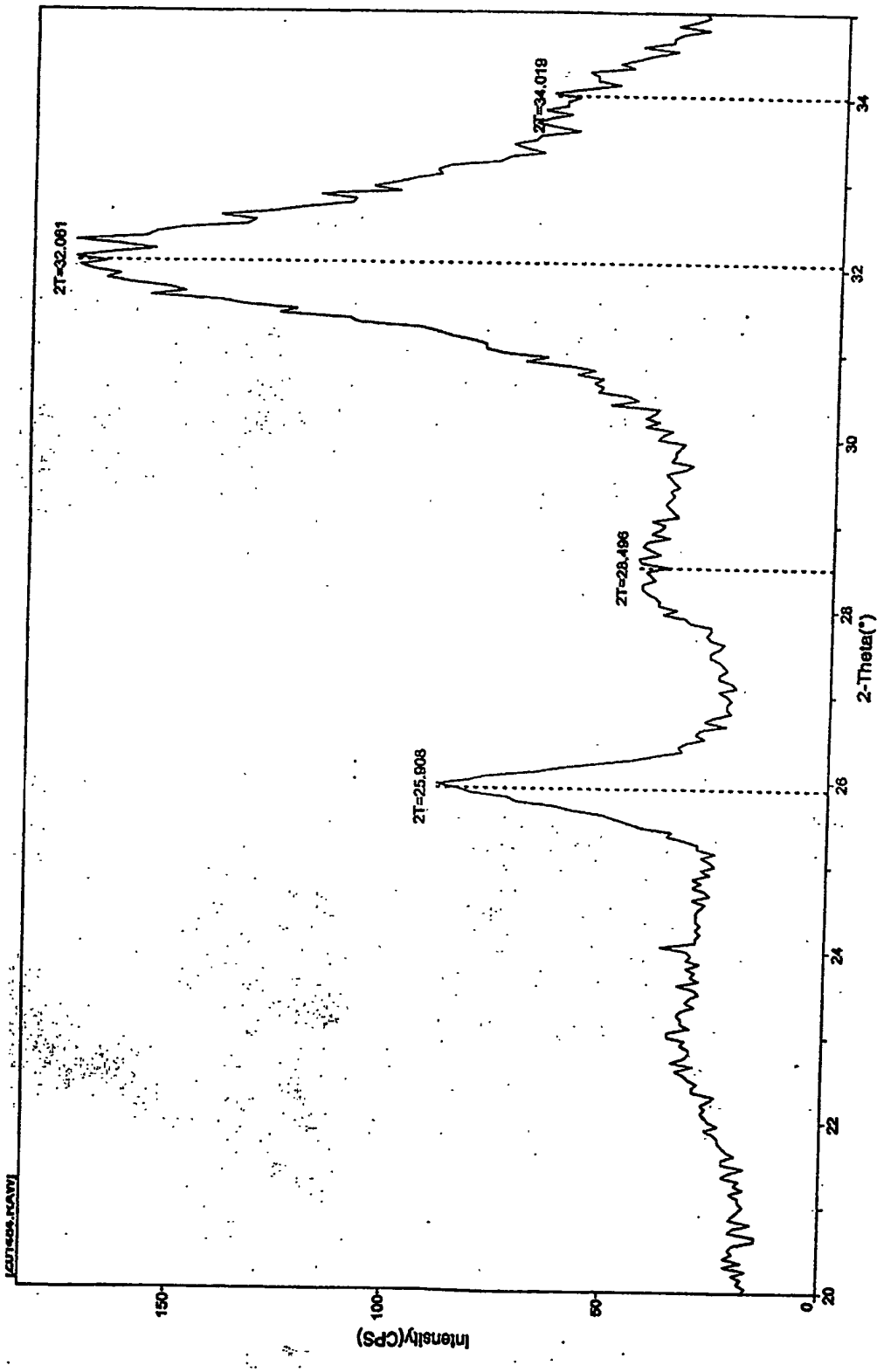
Figure 1C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22
24
26
28
30
32
34

Figure 1D

Figure 1E

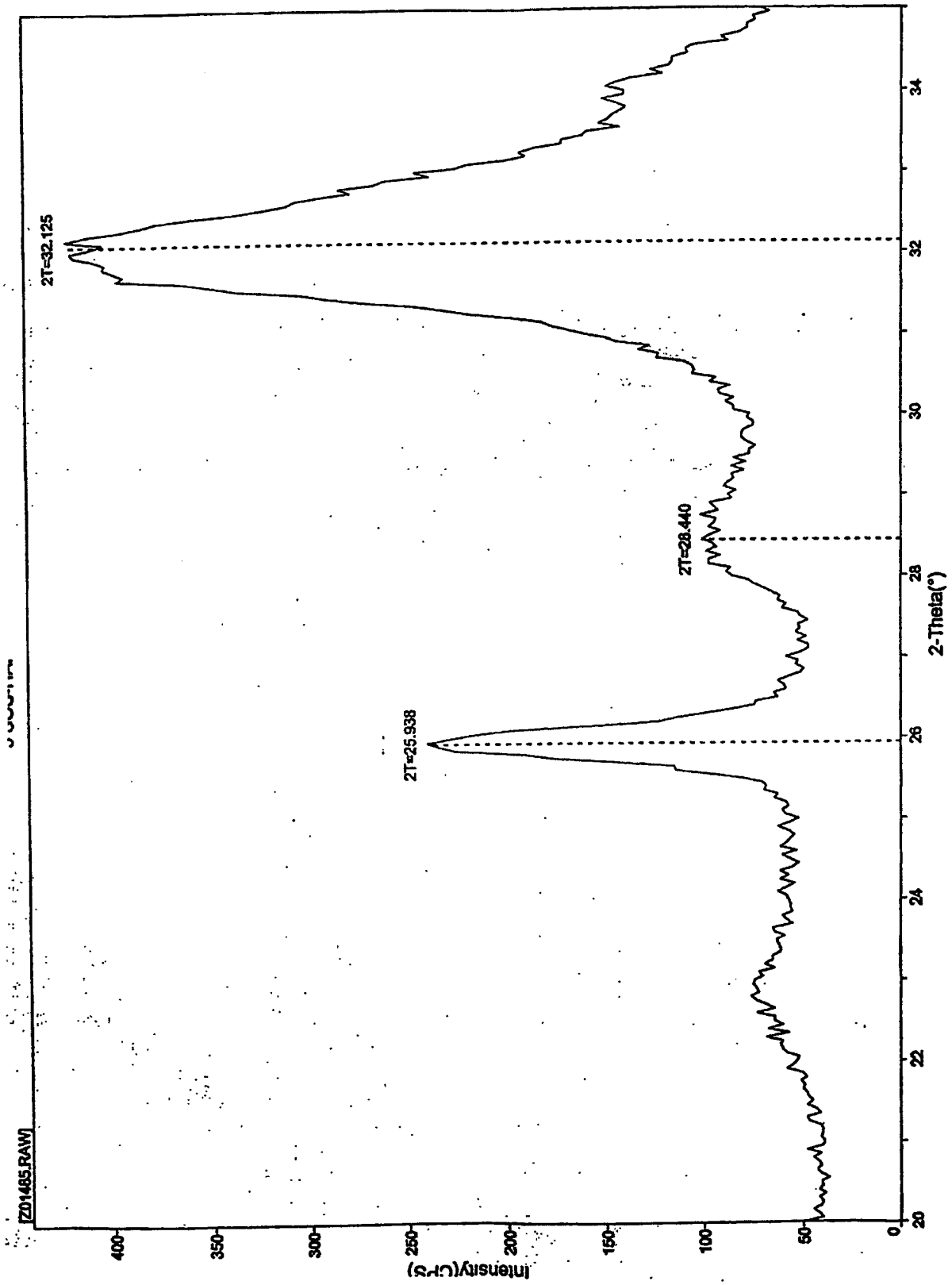

Fig. 2A

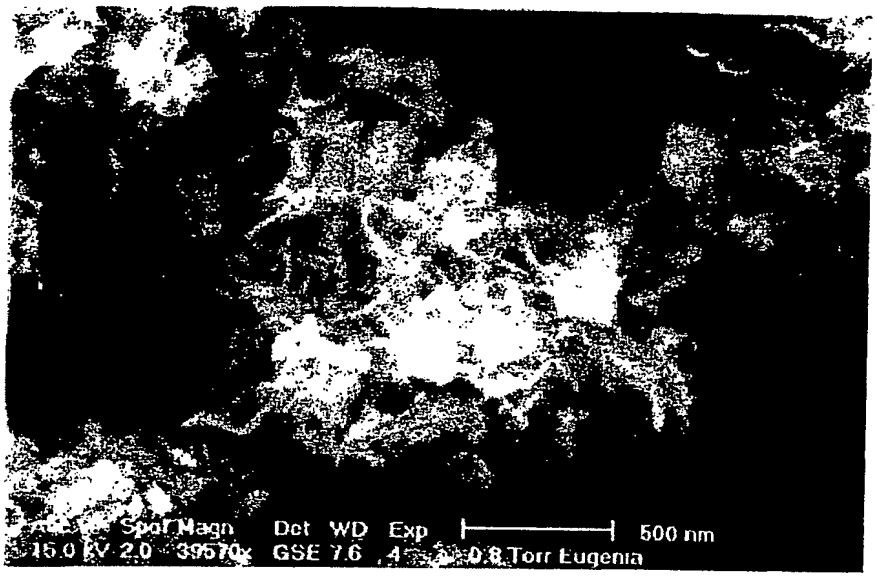

Fig. 2B

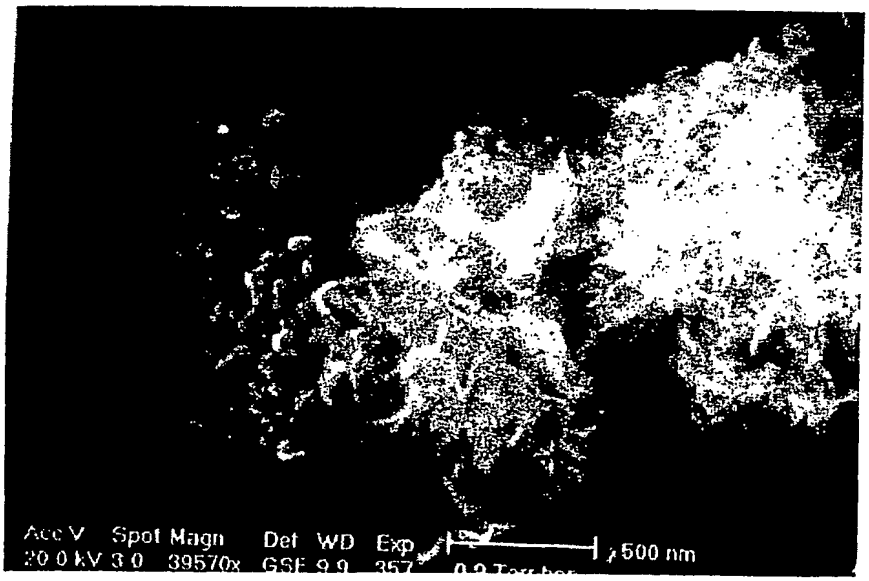

Fig. 2C

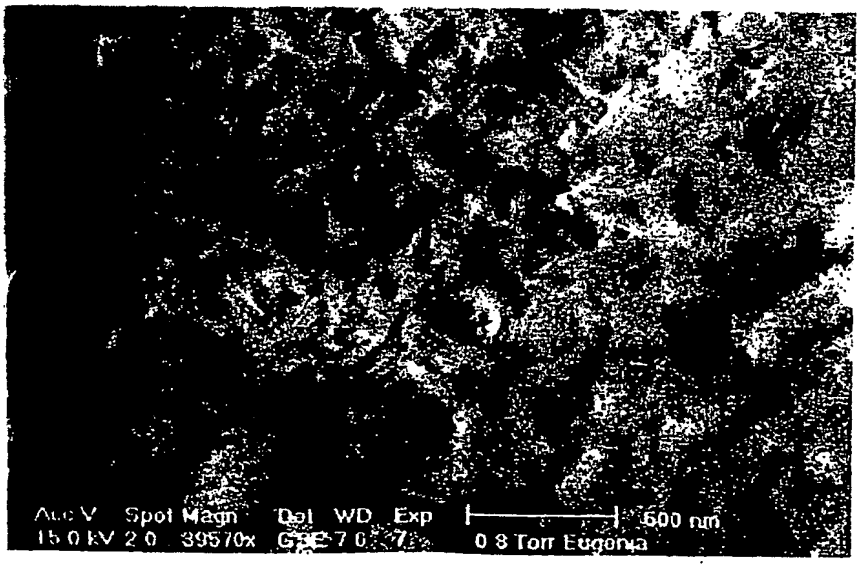

Figure 3A

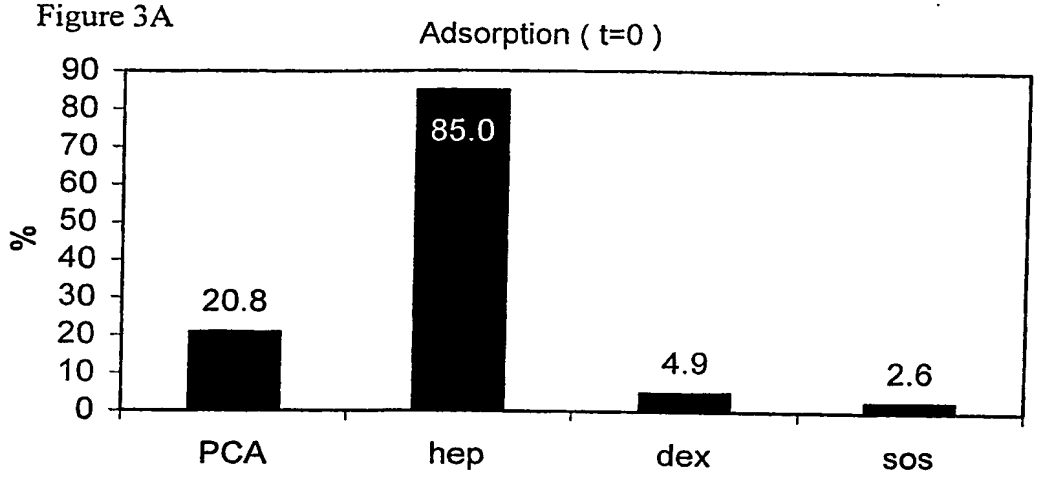

Figure 3B

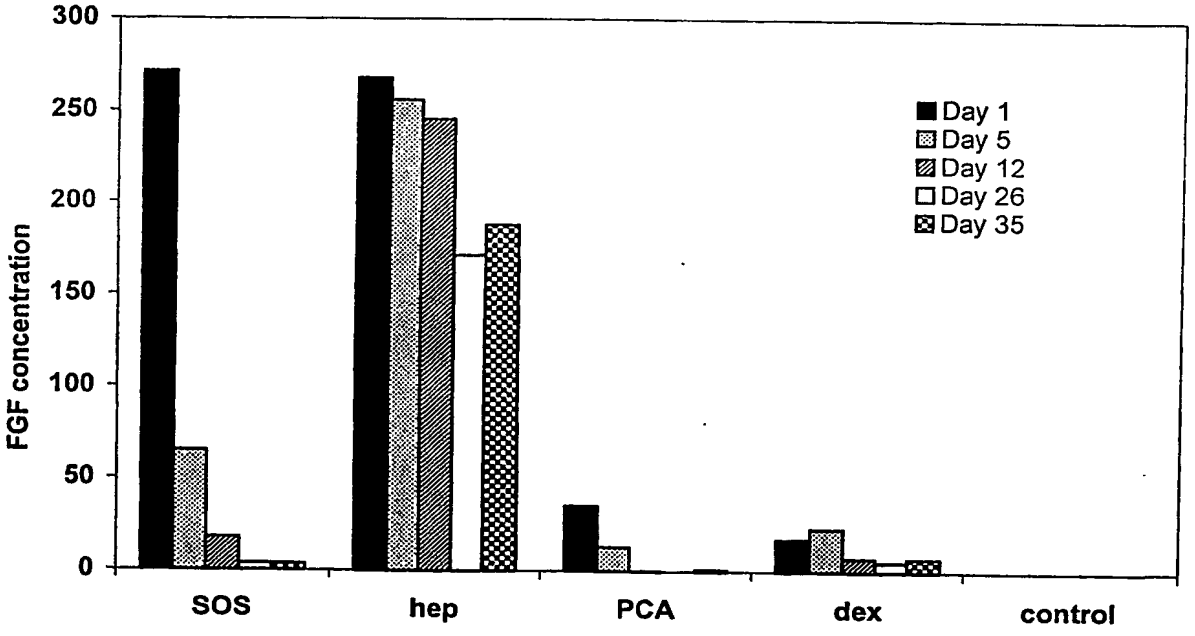

Figure 4A

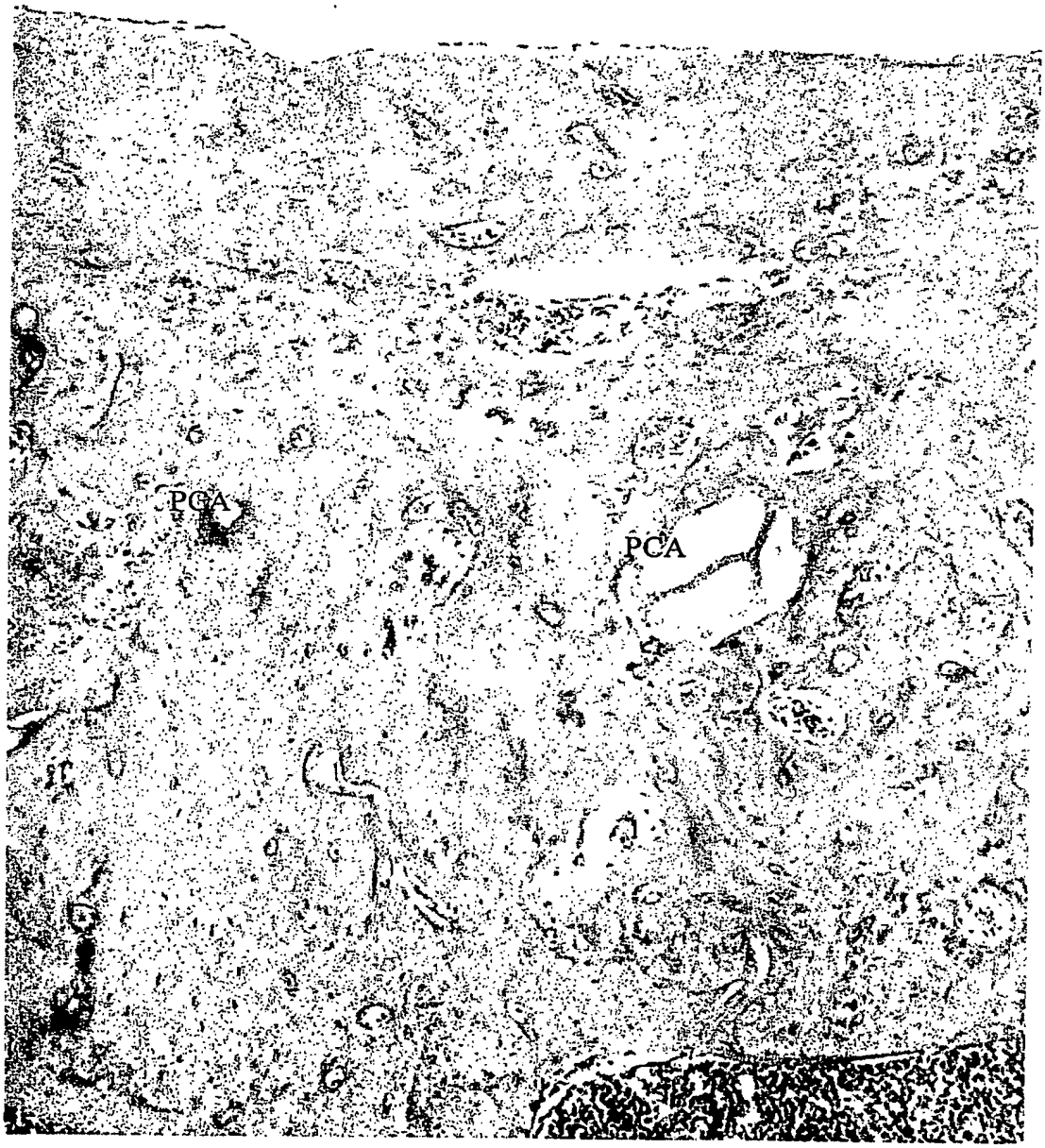

Figure 4B

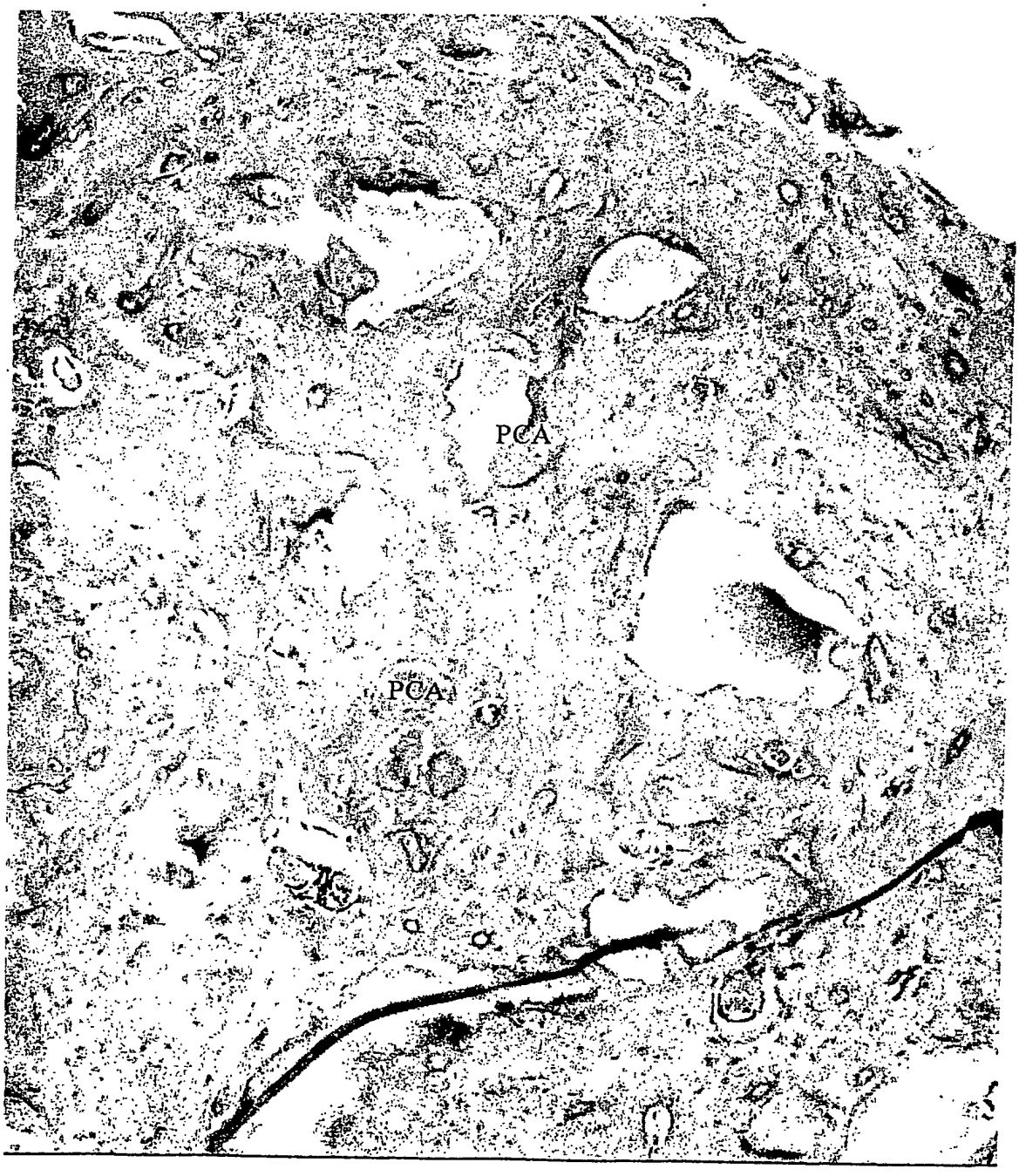

Figure 5A

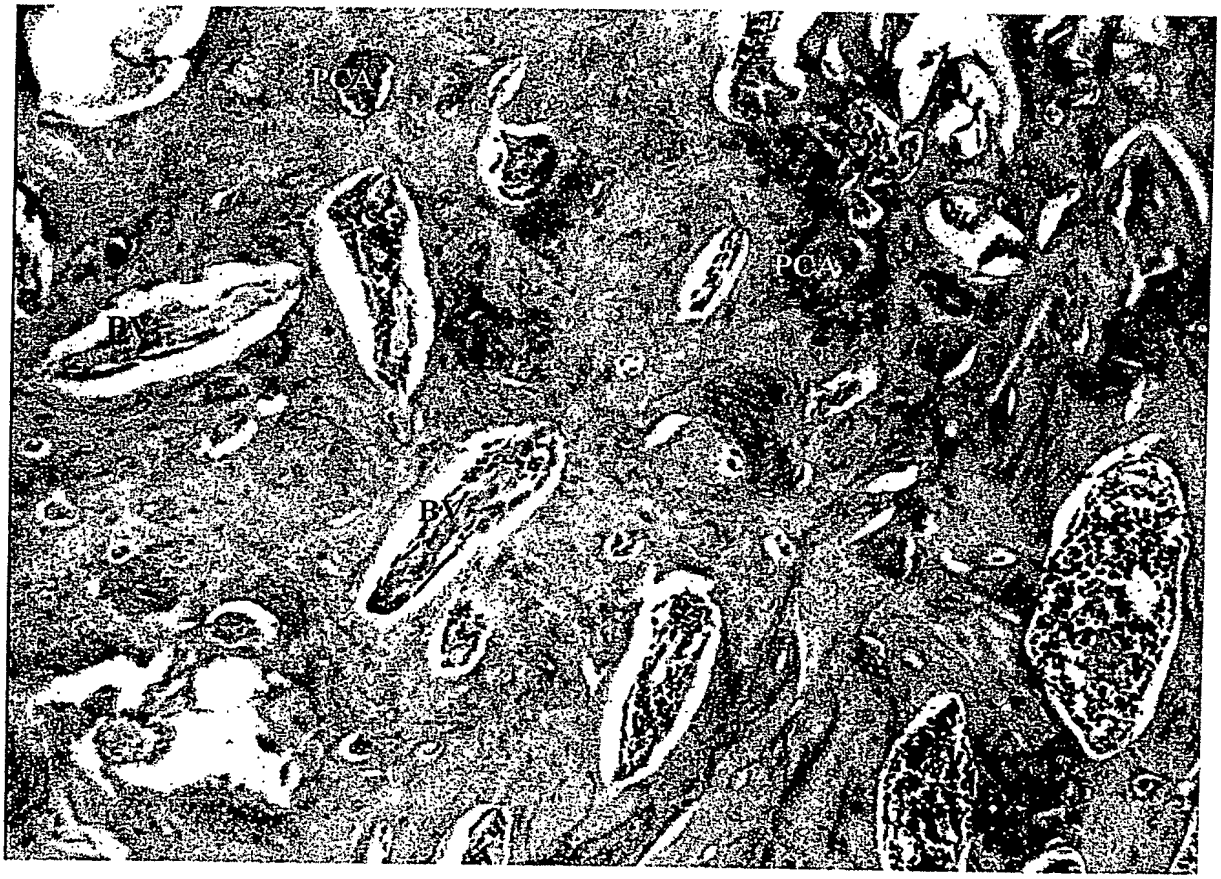

Figure 5B